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13. ABSTRACT ***(Maximum 200 Words)***

E-cadherin is a transmembrane protein that mediates calcium-dependent cell-cell adhesion and plays a role in maintaining the normal phenotype of epithelial cells. Decreased expression of E-cadherin is correlated with increased invasiveness of breast cancer. Inappropriate expression of a non-epithelial cadherin, such as N-cadherin, by an epithelial cell has been shown to down-regulate E-cadherin expression and contribute to cell motility. In this study we explored the possibility that expression of non-epithelial cadherins may be correlated with increased cellular motility and invasion in breast cancer cells. We showed that 1) N-cadherin promotes motility and invasion in breast cancer cells; 2) decreased expression of E-cadherin does not correlate with invasion in breast cancer cells; 3) N-cadherin expression correlates with invasion and motility in breast cancer cells and plays a direct role in promoting motility; 4) forced expression of E-cadherin in invasive, N-cadherin positive cells does not reduce their invasive capacity; 5) forced expression of N-cadherin in non-invasive, E-cadherin-positive cells produces an invasive cell even though these cells continue to express E-cadherin; 6) N-cadherin-dependent motility may be mediated by fibroblast growth factor receptor signaling; and 7) the domain on N-cadherin that is responsible for increased invasion resides in the 4th extracellular repeat domain.

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Introduction

This is the final report for my four year CDA Award. The purpose of this award was to initiate a breast cancer project in my lab. My lab has been focused for many years on the role of cadherin cell adhesion molecules in cellular behavior. Cadherins are cell-surface proteins that play important roles in normal cell-cell interactions. Members of the cadherin family of transmembrane glycoproteins mediate calcium-dependent, homotypic cell-cell adhesion. Numerous studies have implicated E-cadherin as a tumor suppresser protein in carcinomas; i.e., decreased E-cadherin adhesion correlates with a tumor phenotype. The mechanism by which the adhesive function is decreased varies amongst different tumors, but disruption of the function of E-cadherin, regardless of the mechanism, is thought to aid in the formation of a tumor. With this award, I have been able to focus some of my efforts on studying similar questions with regard to human breast cancer cells. Two graduate students focused their efforts on getting the breast cancer project off the ground. Marvin Nieman had been a Ph.D. student in my lab for 4 years and had been studying the effect of dominant-negative cadherins on squamous epithelial cells. He finished up that project and moved on to the breast cancer project. Marvin spent the remainder of his graduate effort on the breast cancer project. He graduated and went on to a post doctoral position at the University of Michigan. Ryan Prudoff was a masters student in the lab who spent a year working with Marvin on a survey of a large number of breast cancer cell lines for expression of cadherins. He finished his masters degree and is currently in medical school at Ohio University. When Marvin graduated, the project was continued by Jae-Boem Kim who mapped the domain on N-cadherin that is responsible for its influence on breast cancer cells. Jae-Boem graduated this past year and is now a post doctoral fellow at University of California, San Francisco. Technical help was provided first by Christine Trapp and then by Bryan Katafiasz. The effort of these individuals has contributed to our understanding of how cadherin-mediated cell interactions may influence the behavior of breast cancer cells. In particular, our lab has shown that N-cadherin, which is not normally expressed by epithelial cells, promotes motility and invasion when expressed by human breast cancer cells.

Body

The cadherins are transmembrane glycoproteins that mediate calcium-dependent homotypic cell-cell adhesion and play an important role in the maintenance of normal tissue architecture. Numerous studies have implicated E-cadherin as a tumor suppresser protein; i.e., decreased E-cadherin function contributes to the development of a tumor. The mechanism by which the adhesive function of E-cadherin is decreased varies from one tumor to another. The decrease may be due to a mutation in the E-cadherin gene or to a mutation in a gene encoding one of the catenins; in other cases the reason for decreased expression or function is not understood. In any case, disruption of the function of E-cadherin is thought to be one step in the development of a carcinoma.

The predominant histological type of breast cancer is infiltrating ductal carcinoma (70%); infiltrating lobular carcinomas are less frequent (6%; Berg and hutten, 1995). Normal breast epithelial cells express E-cadherin and display tight cell-cell contacts with one another. In most cases, lobular cancers are E-cadherin negative; often, but not always, this is due to inactivating mutations in the E-cadherin gene (Berx et al., 1995). However, about 50% of ductal cancers show reduced expression of E-cadherin (Moll et al., 1993) with no evidence for mutations in the gene for E-cadherin (Berx et al., 1995). Thus, at the time we submitted this proposal, no clear correlation had yet been established between mutations in E-cadherin and the altered expression that is so frequently seen in breast carcinomas. A variety of mechanisms other than mutations in E-cadherin could contribute to modulated expression of E-cadherin. This proposal sought funding to explore a unique mechanism that may contribute to the downregulation of E-cadherin in breast carcinomas.

Our hypothesis was that inappropriate expression of another member of the cadherin family plays a role in down-regulation of E-cadherin in some breast carcinomas. This hypothesis stemed from our previous experiments showing that inappropriate expression of N-cadherin by squamous epithelial cells resulted in decreased cell-cell adhesion, increased motility and a fibroblastic morphology (Islam et al., 1996). These properties are typical of metastatic tumor cells. Especially

relevant to our hypothesis, expression of N-cadherin resulted in down-regulation of E-cadherin. We thus wished to explore the hypothesis that expression of an inappropriate cadherin in breast epithelial cells may result in a tumorigenic phenotype.

Our hypothesis was correct and we showed that expression of N-cadherin by breast epithelial cells did indeed influence their behavior. We demonstrated that cells expressing N-cadherin were more motile and more invasive than N-cadherin-negative cells. We implicated fibroblast growth factor receptor signaling in this N-cadherin mediated cell motility and mapped the domain on N-cadherin that influences cell behavior to the extracellular domain.

In addition to N-cadherin, we also examined the effect cadherin-11 has on the behavior of breast epithelial cells. This cadherin is sometimes expressed by breast epithelia so we thought it relevant to include this in our studies. We found that cadherin 11 also increased motility and invasion when expressed by breast epithelial cells. The majority of this work on N-cadherin and cadherin-11 has been published and the citations are included below. Three manuscripts are attached as an appendix.

In addition to the published data, we identified and cloned a novel cadherin that is expressed in human breast epithelial cells. This cadherin has a unique alternatively expressed exon in the extracellular domain. We are in the process of characterizing this cadherin and determining if it plays any role in increased invasive behavior in breast epithelial cells. We are preparing a manuscript describing this new cadherin and hope to publish it within this next year.

During the final year of this project my lab moved from The University of Toledo to The University of Nebraska Medical Center where we are situated within The Eppley Institute for Cancer Research. We are associated with the Breast Cancer program within the Eppley. Unfortunately, we were not allowed by the DOD to transfer the final months of support for this project so it was not completely finished. The money was returned to the DOD by the University of Toledo. We will actively pursue money to complete the work on the novel cadherin once we are settled here at UNMC.

Below is the original Statement Of Work from the proposal and an underlined statement explaining how we have approached each task.

Technical objective 1. Survey cell lines and biopsies:

Tasks 1 &2. Months 1-6: Surveying breast cancer cell lines for E-cadherin expression; survey E-cadherin negative cell lines for expression of N-cadherin, P-cadherin, R-cadherin and Cadherin 5. This was reported in Nieman et al., 1999.

Task 3. Months 7-8. Survey frozen histological sections for expression of the cadherin identified in Task 2. This has been put on hold until we generate a satisfactory antibody.

Task 4. Months 9-12. If we do not identify one specific cadherin in task 2 we will perform PCR using degenerate primers to identify the cadherin of interest. This is irrelevant at this point as we have identified N-cadherin as expressed by invasive breast carcinoma cells. This was published in Nieman et al., 1999.

Task 5. Months 12-18. Prepare antibodies against any other newly identified cadherins if necessary. We identified cadherin 11 as relevant to breast epithelial cellbehavior. An antibody is commercially available against cadherin 11 and we used this in studies published in Nieman et al., 1999.

Technical objective 2. Determine if the expression of inappropriate cadherins contributes to tumorigenesis.

Task 6. Months 12-18: Obtain normal breast cell lines from the Michigan Tissue Bank. Transfect them with X-cadherin and observe the morphology of the transfected cells. X-adherin

turned out to be N-cadherin. We obtained a cell line BT-20 that behaves normally and used this line in studies published in Nieman et al., 1999 and Kim et al., 2000.

Task 7. Months 12-18: Transfect the tumor cells that express X-cadherin with antisense X-cadherin or hammerhead ribozymes directed against X-cadherin and observe the morphology of the transfected cells. We have determined that this is not a feasible experiment. The anti-sense has been transfected and is not effective in down-regulating N-cadherin.

Task 8. Months 18-22: Assay the normal cells, the transfected normal cells from task 6, the tumor cells, the transfected tumor cells from task 7 for motility and invasive characteristics. We have finished these studies and published them (Nieman et al., 1999).

Technical objective 3. Explore the mechanisms that regulate the expression of cadherins in breast tumor cells.

Task 9. Months 22-24: Transform normal breast epithelial cells with ras and determine the levels of expression of E-cadherin and the inappropriate cadherin(s) found in technical objectives 1 and 2 above. Our ideas on this topic have changed since the submission of the original grant. We are pursuing the idea that transformation to the tumorigenic phenotype may be regulated by the FGF receptor. We presented data to this effect in Nieman et al., 1999 and Kim et al., 2000.

Task 10. Month 24: Survey the cell lines that express X-cadherin for expression of erbB-2, EGF receptor and p53. Determine if there is a correlation between any of these markers and expression of X-cadherin or down-regulation of E-cadherin. . Our ideas on this topic have changed since the submission of the original grant. We are pursuing the idea that transformation to the tumorigenic phenotype may be regulated by the FGF receptor. We presented data to this effect in Nieman et al., 1999 and Kim et al., 2000.

Task 11. Months 25-31: Transfect normal breast cells with markers identified in task 10 to determine if overexpression of this marker results in down-regulation of E-cadherin or increased expression of X-cadherin. We transfected cells with the FGF receptor before leaving the University of Toledo. We are in the process of analyzing these cells in our new lab. Unfortunately, the DOD did not allow us to transfer the funds for this project so this experiment is on hold until we secure new funds.

Task 12. Months 24-30: Treat normal breast epithelial cells with estrogen and progesterone to determine if these hormones have an effect on the expression of E-cadherin or other cadherins. Treat normal breast epithelial cells with TGFβ and other TGFβ family members o determine if these hormones have an effect on the expression of E-cadherin or other cadherins. This experiment was done and we did not see any effect.

Task 13. Months 30-36: Analyze the data from task 12 and propose a mechanism for regulation of cadherin expression that can be further explored during the final 6 months of this project. We have focused our efforts on FGF receptor signaling and have proposed a mechanism—partly in Nieman et al., 1999 and partly in Kim et al., 2000.

Educational and trining objectives:

Task 1. Months 1-6: Analyze the literature on breast cancer. Pull together information relevant to this project. This was completed in a timely manner.

Task 2. Months 1-48: Spend some time meeting with Dr. Fearon's lab group to discuss our current collaborative project. Establish new collaborative efforts between our laboratories. I and members of my lab attended a number of lab meetings with the members of Dr. Fearon's lab. My student, Marvin Nieman interacted a lot with this group and did a postdoc in his lab after graduating from my lab. In addition, we interacted significantly with the lab of Dr. Steve Ethier, also at the University of Michigan.

Task 3. Months 36-48: Apply for funds from the NIH to continue this work. The work on cadherins in breast cancer has been continued through my NIH R01. It is up for competitive renewal now.

Key Research Accomplishments

- We showed that human breast cancer cells express N-cadherin and cadherin-11 (Nieman et al., 1999).
- We showed that vimentin expression does not correlate with an invasive phenotype in human breast cancer cells (2000 progress report).
- We identified a new cadherin that is expressed in human breast cancer cells (manuscript in preparation).
- We showed that expression of non-epithelial cadherins is correlated with increased cellular motility and invasion in human breast cancer cells (Nieman et al., 1999).
- We showed that decreased expression of E-cadherin does not correlate with invasion in breast cancer cells (Nieman et al., 2000).
- We showed that N-cadherin plays a direct role in promoting motility (Nieman et al., 1999; Kim et al., 2000).
- We showed that forced expression of N-cadherin in non-invasive, E-cadherin-positive cells produces an invasive cell even though these cells continue to express high levels of E-cadherin (Nieman et al., 1999).
- We showed that N-cadherin-dependent motility may be mediated by fibroblast growth factor receptor signaling (Nieman et al., 1999; Kim et al., 2000).
- We showed that extracellular repeat number 4 of N-cadherin is responsible for increased cell motility in human breast cancer cells (Kim et al., 2000).
- We showed that cadherin-11 behaves in a manner similar to N-cadherin (Nieman et al., 1999)

Reportable Outcomes

- A paper was published in the Journal of Cell Biology. The citation is: Nieman, M.T., Prudoff, R.S., Johnson, K.R. and Wheelock, M.J. 1999. N-cadherin promotes motility in human breast cancer cells regardless of their E-cadherin expression. J. Cell Biol. 147:631-643.
- A paper was published in the Journal of Cell Biology. The citation is: Kim, J.-B., Islam, S., Kim, Y. J., Prudoff, R.S., Sass, K.M., Wheelock, M.J. and Johnson K.R. 2000. N-cadherin extracellular repeat 4 mediates epithelial to mesenchymal transition and increased motility. J. Cell Biol, 151:1193-1205.
- I was asked to write a review for the Journal of Mammary Gland Biology and Neoplasia. The citation is: Wheelock, M.J., Peralta Soler, A. and Knudsen, K.A. 2001. Cadherin junctions in mammary tumors. J. Mammary Gland Biology and Neoplasia 6:275-285.
- This work was presented at the 89th meeting of the American Association for Cancer Research in 1999.
- This work was presented as an invited talk at the Biological Structure and Gene Expression Gordon Conference in 1999.
- This work was presented as a platform talk at the American Society for Cell Biology Meeting in 1999.
- This work was presented as an invited talk at the International Bat-Sheva de Rothschild Seminar Weizmann Institute, Rehovot, Israel in 1999.
- This work was presented at the Era of Hope DOD Breast Cancer Meeting in 2000.
- This work was presented as an invited talk at the Cell contact and adhesion Gordon Conference in 2001.

- ❖ Marvin Nieman graduated with a Ph.D. in biology based on work supported on this project.
- ❖ Ryan Prudoff graduated with a masters degree in biology based on work supported on this project.
- ❖ Jae Boem Kim graduated with a Ph.D. in biology based in part on work supported on this project.
- ❖ Several cell lines were generated by transfecting cadherins into breast cancer cells.
- ❖ Dr. Marvin Nieman was granted a position at the University of Michigan as a postdoctoral fellow based on his work as a Ph.D. student in my laboratory.
- ❖ Dr. Jae Boem kim was granted a position at the University of California San Francisco as a postdoctoral fellow based on his work as a Ph.D. student in my laboratory.
- ❖ Ryan Prudoff was accepted into medical school in part due to his work on this project.

Conclusions

Our laboratory previously showed that expression of different cadherin family members by squamous epithelial cells markedly effected morphology (Islam et al., 1996), i.e., when oral squamous epithelial cells expressed N-cadherin, they converted to a fibroblastic phenotype concurrent with decreased cell-cell adhesion. Thus, when we turned our attention to breast cancer cells for the present study, we were interested not only in the expression of various cadherins by these cells, but also in whether these cadherins influenced the morphology of the cells. We were not surprised to find that breast cancer cells endogenously expressing N-cadherin displayed a fibroblastic phenotype with tenuous cell-cell contacts, while breast cancer cells endogenously expressing E-cadherin displayed a typical epithelial morphology. We were, however, surprised to find that transfection of N-cadherin into the E-cadherin-expressing BT-20 breast cancer cell line had no effect on morphology even though it had a dramatic effect on cell behavior. Equally surprising was the fact that forced expression of E-cadherin had no effect on the morphology of the fibroblastic N-cadherin-expressing MDA-MB-435 cells. Thus, the breast cancer cell lines examined in this study behaved very differently from the oral squamous epithelial lines that we previously characterized. These results were published in Nieman et al., 1999 which is in the appendix.

In the four years of this study, we have demonstrated that N-cadherin expression in human breast carcinoma cells promotes an invasive phenotype. By transfecting the non-invasive BT-20 cells with these non-epithelial cadherins, we have provided evidence for a direct role of these cadherins in cell motility and invasion. Previous studies have correlated the expression of N-cadherin with invasion in breast cancer cells. However, in this study we took the important next step and used transfection studies to show that a previously non-invasive cell could be converted to an invasive cell by expression of N-cadherin. The BT-20 breast cancer cell line provided an important tool for these studies since they did not down-regulate E-cadherin when forced to express N-cadherin. Thus, we can conclude that, even in cells expressing high levels of E-cadherin, N-cadherin can promote motility, suggesting that, in this regard, N-cadherin is "dominant" over E-cadherin. A study by Sommers et al. (1994) supports this idea. These authors showed that transfection of E-cadherin into the E-cadherin-negative breast cancer cell lines BT549 and HS578 did not decrease the invasive capacity of these cells. These authors suggested that the transfected E-cadherin was not functional; however, these authors were unaware of the fact that the BT549 and HS578 cell lines express N-cadherin.

One puzzling aspect of cell lines derived from metastatic tumors is that they often express E-cadherin and appear to be relatively normal epithelial cells. A possibility suggested by our results is that these cells upregulated the expression of N-cadherin during the process of metastasis. Our results suggest that expression of N-cadherin would confer on these cells the capacity to invade even though they continued to express E-cadherin. In this regard, expression of an inappropriate

cadherin like N-cadherin (or other related cadherins) may be a better gauge of the clinical state of a tumor than is decreased expression of E-cadherin.

Understanding the mechanism by which N-cadherin promotes motility in epithelial cells is important if we are to develop treatments that will decrease the invasiveness of tumor cells. A number of studies have shown that epithelial cells can be induced to scatter in response to growth factors such as hepatocyte growth factor and members of the fibroblast growth factor, epidermal growth factor and transforming growth factor families (Vallés et al., 1990; Blay and Brown, 1985; Geimer and Bade, 1991; Miettinen et al., 1994; Behrens et al., 1991; Gherardi and Stoker 1991; Rosen et al., 1991; Savagner et al., 1994; Savagner et al., 1997). Walsh, Doherty and co-workers have established through extensive studies on FGF receptor and cell adhesion molecules that N-cadherin and the FGF receptor cooperate to induce neurite outgrowth in cerebellar neurons (reviewed in Doherty and Walsh, 1996; Walsh and Doherty, 1997). These authors have proposed a scheme for activation of the kinase activity of the FGF receptor through cis interactions with N-cadherin via an HAV domain in the FGF receptor and an HAV interaction domain in the fourth extracellular domain of N-cadherin (Doherty and Walsh, 1996). In addition, it has been proposed that the cadherins form lateral dimers in the plane of the membrane (Shapiro et al., 1995; Takeda et al., 1999), which could result in dimerization of the FGF receptor and subsequent activation of the signal transduction pathway. We based the studies presented herein on the model presented by Walsh and Doherty and proposed that interaction of N-cadherin with the FGF receptor in N-cadherin-expressing epithelial cells may result in increased motility similar to that seen by treating epithelial cells with growth factors. To test this hypothesis, we interfered with the N-cadherin-dependent FGF receptor signal transduction pathway proposed by Walsh and Doherty by inhibiting a downstream enzyme, diacylglycerol lipase. We showed that inhibiting diacylglycerol lipase decreased motility of N-cadherin-expressing cells in a dose-dependent manner while having no effect on the motility of N-cadherin-negative cells. Thus, our data strongly support the notion that N-cadherin promotes motility in breast cancer cells by activating growth factor receptor signal transduction pathways. These results were published in Nieman et al., 1999 which is in the appendix.

To determine which domain of N-cadherin influences motility, we constructed two chimeric cadherins. The first consisted of the extracellular and transmembrane domains of E-cadherin connected to the cytoplasmic domain of N-cadherin (E/N-cadherin). The second chimera consisted of the extracellular and transmembrane domains of N-cadherin connected to the cytoplasmic domain of E-cadherin (N/E-cadherin). See Kim et al., 2000 in the appendix for a diagram of the chimeric cadherins. Our goal was to test E/N-cadherin and N/E-cadherin for effects on cellular behavior using the human breast cancer cell line BT20 that changes from a relatively non-motile to a highly motile cell when transfected with N-cadherin (Nieman et al., 1999). Before testing the effect our chimeric cadherins had on the behavior of cells, we showed that each chimera was a functional adhesion molecule by transfecting them into cadherin-negative cells and showing that they associated with catenins in an immunoprecipitation assay, localized to the cell surface and mediated cell aggregation (see Figure 1 Kim et al., 2000 in the appendix). Thus, both E/N-cadherin and N/E-cadherin functioned as adhesion molecules in a manner similar to E-cadherin or N-cadherin.

E/N-cadherin and N/E-cadherin were transfected into BT20 cells and analyzed for their ability to induce motility. To our surprise, N/E-cadherin was as efficient as intact N-cadherin at inducing motility in BT20 cells whereas E/N cadherin did not significantly alter the motile characteristics of BT20 cells. Thus, we showed that the extracellular domain of N-cadherin is responsible for the epithelial to mesenchymal transition in squamous epithelial cells and for increased motility in breast cancer cells. These results were published in Kim et al., 2000 which is in the appendix.

To investigate further the extracellular domain of N-cadherin and its role in cell motility, we constructed additional chimeric cadherins, starting with N/E-cadherin and moving the boundary between N- and E-cadherin progressively toward the N terminus. When these chimeras were

transfected into BT20 cells, we were able to narrow down the motility-inducing region to extracellular domain 4 and we now propose that this portion of N-cadherin interacts with the FGF receptor to induce motility in breast cancer cells. These results were published in Kim et al., 2000 which is in the appendix.

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Appendices

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N-Cadherin Promotes Motility in Human Breast Cancer Cells Regardless of their E-Cadherin Expression

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Abstract. E-cadherin is a transmembrane glycoprotein that mediates calcium-dependent, homotypic cell–cell adhesion and plays a role in maintaining the normal phenotype of epithelial cells. Decreased expression of E-cadherin has been correlated with increased invasiveness of breast cancer. In other systems, inappropriate expression of a nonepithelial cadherin, such as N-cadherin, by an epithelial cell has been shown to downregulate E-cadherin expression and to contribute to a scattered phenotype. In this study, we explored the possibility that expression of nonepithelial cadherins may be correlated with increased motility and invasion in breast cancer cells. We show that N-cadherin promotes motility and invasion; that decreased expression of E-cadherin does not necessarily correlate with motility or invasion; that N-cadherin expression correlates both with invasion and motility, and likely plays a direct role in promoting motility; that forced expression of E-cadherin in invasive, N-cadherin–positive cells does not reduce their motility or invasive capacity; that forced expression of N-cadherin in noninvasive, E-cadherin–positive cells produces an invasive cell, even though these cells continue to express high levels of E-cadherin; that N-cadherin–dependent motility may be mediated by FGF receptor signaling; and that cadherin-11 promotes epithelial cell motility in a manner similar to N-cadherin.

Key words: N-cadherin • E-cadherin • breast cancer • motility • fibroblast growth factor receptor

CADHERINS constitute a family of transmembrane glycoproteins that mediate calcium-dependent homotypic cell–cell adhesion and play an important role in the maintenance of normal tissue architecture. The cadherin intracellular domain interacts with several proteins, collectively called catenins, that link cadherins to the actin cytoskeleton (reviewed in Wheelock et al., 1996). This linkage is required for full cadherin adhesive activity. Either β-catenin or plakoglobin binds directly to the cadherin and to α-catenin, whereas α-catenin links directly and indirectly to actin (Aberle et al., 1994; Nagafuchi et al., 1994; Stappert and Kemler, 1994; Knudsen et al., 1995; Rimm et al., 1995; Nieset et al., 1997; Watabe-Uchida et al., 1998). Their ability to simultaneously self-associate and link to the actin cytoskeleton enables cadherins to mediate both the cell recognition required for cell sorting and the strong cell–cell adhesion needed to form tissues.

In addition to their structural role in the adherens junction, catenins are thought to regulate the adhesive activity of cadherins. For example, phosphorylation of β-catenin in Src transformed cells may contribute to the nonadhesive phenotype of these cells (Matsuyoshi et al., 1992; Hamaguchi et al., 1993). In addition, $p120^{ctn}$, originally identified as a Src substrate and subsequently shown to bind to the cytoplasmic domain of cadherins, has been suggested to play a role in regulating the adhesive activity of cadherins (Reynolds et al., 1994; Daniel and Reynolds, 1995; Shibamoto et al., 1995; Aono et al., 1999; Ohkubo and Ozawa, 1999).

Numerous studies have demonstrated the importance of the E-cadherin/catenin complex in maintaining the normal phenotype of epithelial cells. Early studies showed that inhibiting E-cadherin activity with function-perturbing antibodies altered the morphology of MDCK cells and conferred upon them the ability to invade both collagen gels and embryonic chicken heart tissue (Behrens et al., 1989; Chen and Öbrink, 1991). In addition, invasive, fibroblast-like carcinoma cells could be converted to a noninvasive phenotype by transfection with a cDNA encoding E-cadherin (Frixen et al., 1991). Moreover, E-cadherin expression is downregulated or lost in epithelial tumors from various tissues, including stomach, colon, head and neck, bladder, prostate, and breast (Schipper et al., 1991; Bringuier et al., 1993; Dorudi et al., 1993; Mayer et al., 1993; Oka et al., 1993; Umbas et al., 1994).

It has been suggested that alterations in cadherin function may be a critical step in the development of breast cancers. A survey of 18 cell lines derived from breast carci-

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nomas showed that ten lines failed to express detectable levels of E-cadherin, and two other lines failed to express α-catenin (Pierceall et al., 1995). Other studies have identified breast tumor cell lines with mutations in the E-cadherin gene (Berx et al., 1995), or with changes in the levels of expression or in the phosphorylation state of β-catenin or plakoglobin (Sommers et al., 1994). Surveys of breast cancer tissue make an equally compelling case for the involvement of E-cadherin in the formation or progression of breast tumors, and clinical studies have shown that loss of E-cadherin correlates with metastatic disease and poor prognosis (Gamello et al., 1993; Moll et al., 1993; Oka et al., 1993; Rasbridge et al., 1993; Berx et al., 1996; Guriec et al., 1996).

In vitro studies support the role of E-cadherin as an invasion suppressor gene. For example, forced expression of E-cadherin in rat astrocytoma cells suppressed motility (Chen et al., 1997). Likewise, transfection of invasive E-cadherin–negative breast or prostate cell lines with mouse E-cadherin resulted in cells that were less invasive in in vitro assays (Frixen et al., 1991; Luo et al., 1999). When treated with function blocking E-cadherin antibodies, the transfected cells returned to an invasive phenotype, thus implicating E-cadherin as an invasion suppressor (Frixen et al., 1991).

Although a number of studies with breast carcinoma cell lines have shown that loss of E-cadherin generally results in an invasive phenotype, important exceptions have been reported. In one study, two E-cadherin–negative cell lines were shown to be noninvasive (Sommers et al., 1991). These authors suggested that in order for E-cadherin–negative cells to be invasive, they must also express vimentin.

In another study, Sommers et al. (1994) showed that transfection of E-cadherin into the invasive breast cancer cell lines, BT549 and HS578t, altered neither the morphology nor the invasive behavior of these cells. These authors speculated that the transfected E-cadherin may not be fully functional in these cells, due to altered posttranslational modification of the cadherin-associated proteins β-catenin, α-catenin, or plakoglobin.

It has been suggested that, unlike E-cadherin, N-cadherin may promote motility and invasion in carcinoma cells. For example, Hazan et al. (1997) reported that expression of N-cadherin by breast carcinoma cells correlated with invasion, and suggested that invasion was potentiated by N-cadherin–mediated interactions between the breast cancer cells and stromal cells. A study conducted in our laboratory suggested that N-cadherin may play a more direct role in the process of invasion and may actually promote invasion by inducing a scattered phenotype when expressed by oral squamous cell carcinoma-derived cells (Islam et al., 1996). In this study, forced expression of N-cadherin resulted in downregulation of endogenous E- and P-cadherins, making it impossible to separate the motility-promoting effects of N-cadherin from the motility-suppressing activity of E-cadherin. In contrast, it has been suggested that N-cadherin promotes contact inhibition in normal skeletal muscle myoblasts and, in so doing, inhibits migration upon contact, but does not suppress motility in subconfluent cells (Huttenlocher et al., 1998).

Thus, the information in the literature concerning the role cadherins may play in tumor cell invasion is inconclusive and even contradictory, prompting us to revisit the question using new reagents generated by our laboratory to examine both previously studied and newly derived breast cancer cell lines. The data presented in this paper indicate: decreased expression of E-cadherin does not necessarily correlate with invasion in breast cancer cells; N-cadherin expression correlates both with invasion and motility in breast cancer cells, and likely plays a direct role in promoting motility; forced expression of E-cadherin in invasive, N-cadherin–positive cells does not reduce their motility or invasive capacity; forced expression of N-cadherin in noninvasive, E-cadherin–positive cells produces an invasive cell, even though these cells continue to express high levels of E-cadherin; the data suggest that N-cadherin-mediated cell motility may be stimulated by FGF receptor signaling; and other cadherins, such as cadherin-11, may promote motility in epithelial cells in a manner similar to N-cadherin.

Materials and Methods

Cells

Breast carcinoma cell lines were obtained from American Type Culture Collection (ATCC) and maintained in DME with 10% FBS (SKBr3, MDA-MB-435, MDA-MB-436, BT-549, and Hs578t) or MEM with 10% FBS (MDA-MB-453 and BT-20). The cell lines MCF-7 and MDA-MB-231 were obtained from Dr. Mary J.C. Hendrix (University of Iowa, Iowa City, IA) and maintained in DME with 10% FBS. The cell lines SUM 159PT and SUM 149 were kindly provided by Dr. Steve Ethier and generated by the University of Michigan Human Breast Cell/Tissue Bank and Data Base. They were maintained in Ham's F-12 with 5% FBS supplemented with insulin (5 mg/ml) and hydrocortisone (1 mg/ml). The cell line SUM 1315 was obtained from the same source and maintained in Ham's F-12 with 5% FBS supplemented with insulin (5 mg/ml) and EGF (10 ng/ml). HT1080 cells were obtained from ATCC and maintained in DME with 10% FBS.

Transfections

To transfect MDA-MB-435 with E-cadherin, the calcium phosphate transfection kit (Stratagene) was used, according to manufacturer's protocol. For electroporations (BT-20 cells), 10^6 cells were washed with PBS and resuspended in electroporation buffer (120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4, 10 mM KH_2PO_4, 25 mM Hepes, 2 mM EGTA, 5 mM $MgCl_2$) supplemented with 2 mM ATP and 5 mM glutathione. After a 5 min incubation on ice, the cells were electroporated at 500 μF and 380 V in a Bio-Rad gene pulser. Cells were immediately plated in a 100-mm dish in complete medium. Floating cells were removed and fresh medium was added 24 h after electroporation; puromycin was added to the culture for selection of clones 48 h after electroporation.

Clones and Vectors

For transfection of N-cadherin, a restriction fragment containing nucleotides 442–3362 (GenBank/EMBL/DDBJ accession number S42303; a kind gift of Dr. Avri Ben-Ze'ev, The Weizmann Institute of Science, Israel) was ligated into the expression vector pLK-pac (Islam et al., 1996). The E-cadherin construct has been described previously (Lewis et al., 1997). The human cadherin-11 cDNA was provided by Drs. S. Takashita and A. Kudo (Tokyo Institute of Technology, Japan; accession number D21254; Okazaki et al., 1994).

Antibodies and Reagents

Unless otherwise stated, all reagents were from Sigma Chemical Co. Rabbit polyclonal antibodies (Jelly) against human E-cadherin extracellular domain (Wheelock et al., 1987), and mouse mAbs against E-cadherin

(HECD1; a kind gift of Dr. Masatoshi Takeichi, Kyoto University, Kyoto, Japan) and N-cadherin (13A9; Knudsen et al., 1995, Sacco et al., 1995), have been described previously. The mouse mAb against β-catenin (6E3) was made as described by Johnson et al. (1993). The mouse mAbs against cadherin-11 were kindly provided by Dr. Marion Bussemakers (University Hospital Nijmegen, The Netherlands). The diacylglycerol lipase inhibitor, RHC80267, was purchased from BIOMOL.

Extraction of Cells

Monolayers of cells were washed with PBS at room temperature and extracted on ice with 2.5 ml/75 cm^2 flask 10 mM Tris acetate, pH 8.0, containing 0.5% NP-40 (BDH Chemicals Ltd.), 1 mM EDTA, and 2 mM PMSF. The cells were scraped, followed by vigorous pipetting for 5 min on ice. Insoluble material was removed by centrifugation at 15,000 *g* for 10 min at 4°C. Cell extracts were resolved on 7% SDS-PAGE as described (Lewis et al., 1994), transferred electrophoretically to nitrocellulose, and immunoblotted as described (Wheelock et al., 1987) using primary antibodies followed by ECL, according to the manufacturer's protocol (Pierce Chemical Co.). For the purpose of loading equal amounts of protein onto SDS-PAGE, quantification was done using the BioRad protein assay reagent according to the manufacturer's protocol.

Immunofluorescence and Microscopy

Cells were grown on glass coverslips, fixed with Histochoice (Amresco), washed three times with PBS, and blocked for 30 min with PBS supplemented with 10% goat serum. Coverslips were exposed to primary antibodies for 1 h, washed three times with PBS, and exposed to species-specific antibodies conjugated to FITC or rhodamine for 1 h. Cells were viewed using a Zeiss Axiophot microscope equipped with the appropriate filters, and photographed using Kodak T-MAX 3200 film. Living cells were viewed using a Zeiss Axiovert microscope and photographed using Kodak T-MAX 400 film.

In Vitro Invasion Assays and Motility Assays

For motility assays, 5×10^5 cells were plated in the top chamber of noncoated polyethylene teraphthalate (PET) membranes (6-well insert, pore size 8 mm; Becton Dickinson). For in vitro invasion assays, 3×10^4 cells were plated in the top chamber of Matrigel-coated PET membranes (24-well insert, pore size 8 mm; Becton Dickinson). In motility and invasion assays, 3T3 conditioned medium was used as a chemoattractant in the lower chamber. The cells were incubated for 24 h and those that did not migrate through the pores in the membrane were removed by scraping the membrane with a cotton swab. Cells transversing the membrane were stained with Diff-Quick (Dade). Cells in ten random fields of view at 100× were counted and expressed as the average number of cells/field of view. Three independent experiments were done in each case. The data were represented as the average of the three independent experiments with the SD of the average indicated. When cells were induced with dexamethasone to express a transgene, the control cells were treated with the same level of dexamethasone. To inhibit FGF receptor signaling, cells were treated with RHC80267 (which inhibits the activity of diacylglycerol lipase) at a concentration of 10–40 μg/ml 3T3 conditioned culture medium during the 24 h of the assay.

Results

Expression of Cadherins by Breast Cancer Cells

E-cadherin has been termed a tumor suppressor, mainly because cells derived from E-cadherin–negative epithelial tumors tend to be invasive, whereas cells derived from E-cadherin–positive tumors tend not to be. In the case of cells derived from breast carcinomas, the majority of E-cadherin–negative cells are invasive (Sommers et al., 1991, 1994; Pierceall et al., 1995). However, an increasing number of exceptions to this rule are becoming evident. Our laboratory has recently shown that expression of an inappropriate cadherin by an oral squamous carcinoma cell line influences expression of E-cadherin and the cellular phenotype (Islam et al., 1996). This observation led us to hypothesize that the invasiveness of some breast cancer cells may be due to an increase in the expression of an inappropriate cadherin, possibly N-cadherin, rather than to a decrease in the expression of E-cadherin. To test this hypothesis, we surveyed a large number of cell lines, many of which had been characterized previously, for expression of E- and N-cadherin. The data, which are summarized in Table I, supported our notion that invasiveness is correlated with N-cadherin expression, rather than lack of E-cadherin expression.

Table I. Cadherin Expression in Breast Carcinoma Cell Lines

Cell line	E-cadherin	N-cadherin	P-cadherin	Cadherin-11	β-Catenin	Motility
MCF-7	+*‡	−*	−*	−*	+*‖¶	No*‡
BT-20	+*	−*	+*	−*	+*	No*
SUM149	+*	−*	+*	−*	+*	No*
SKBr3	−*‡	−*	−*	−*	−*‖¶	No*‡
MDA-MB-453	−*‡	−*	−*	−*	−*‖¶	No‡
SUM1315	−*	−*	+*	±*	+*	No*
MDA-MB-435	−*‡	+*	−*	−*	+*¶	Yes§
MDA-MB-436	−*‡	+*	−*	−*	+*‖¶	Yes‡
BT549	−*‡	+*	+*	−*	+*‖¶	Yes‡
Hs578t	−*†	+*	−*	−*	+*‖¶	Yes*‡
SUM159PT	−*	+*	−*	−*	+*	Yes*
MDA-MB-231	−*	−*	−*	+*†	+*	Yes*†

*Current study; ‡Sommers et al., 1991; §Frixen et al., 1991; ‖Sommers et al., 1994; ¶Pierceall et al., 1995; †Pishvaian et al., 1999.

Fig. 1 is an immunoblot of extracts of the cell lines presented in Table I. Equal amounts of protein were loaded in each lane. The samples were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted for E-, N-, or P-cadherin, cadherin-11, and β-catenin. Fig. 2 presents phase micrographs of the living cells to compare the

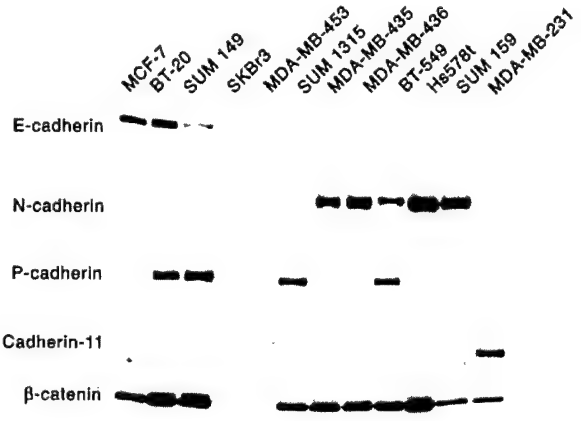

Figure 1. Cadherin and β-catenin expression in breast carcinoma cell lines. Confluent monolayers of MCF-7, BT-20, SUM 149, SKBr3, MDA-MB-453, SUM 1315, MDA-MB-435, MDA-MB-436, BT-549, Hs578t, SUM 159PT, or MDA-MB-231 were extracted with NP-40. 20 μg total protein from each cell extract was resolved by SDS-PAGE, transferred to nitrocellulose, and blotted with antibodies against E-cadherin (HECD1), N-cadherin, P-cadherin, cadherin-11, or β-catenin.

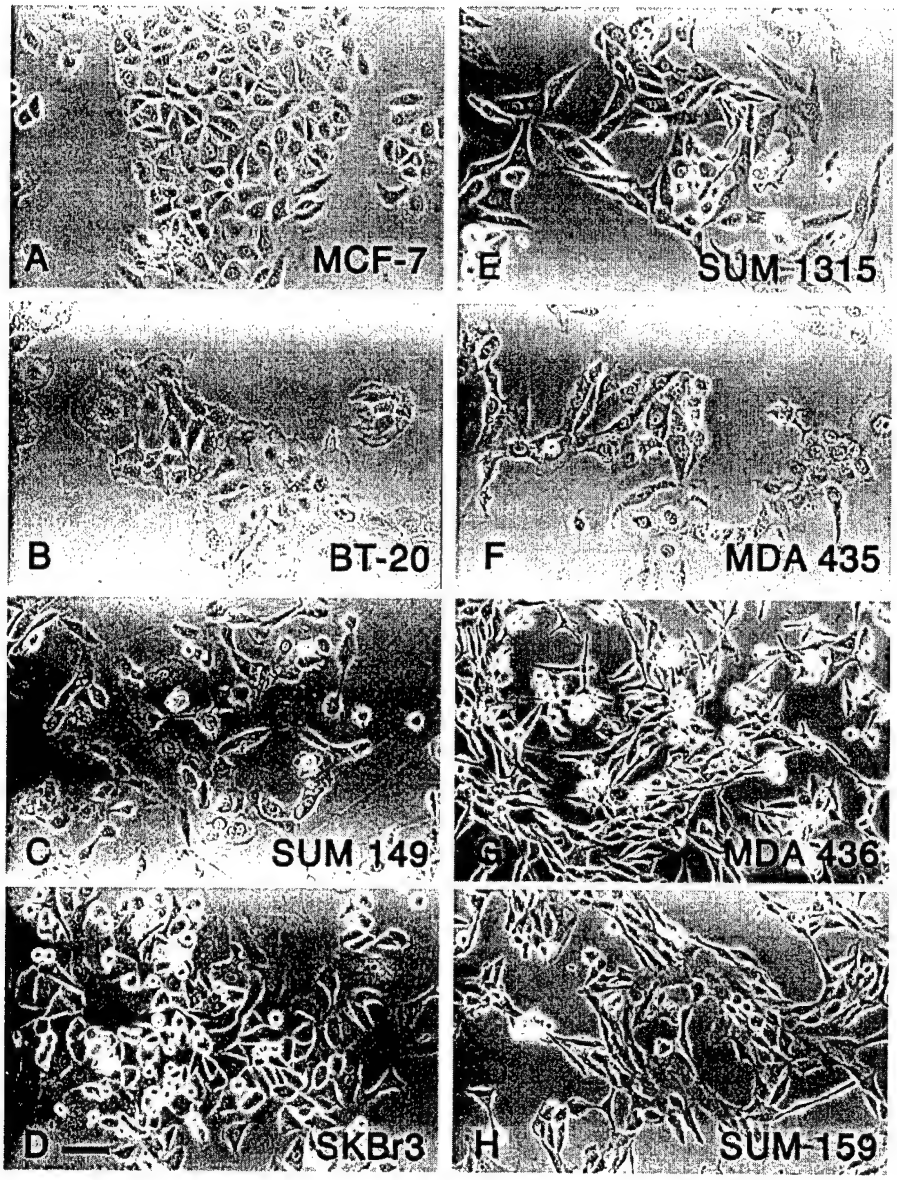

Figure 2. Morphological analysis of breast cancer cell lines. Living monolayers of MCF-7 (A), BT-20 (B), SUM 149 (C), SKBr3 (D), SUM 1315 (E), MDA-MB-435 (F), MDA-MB-436 (G), or SUM 159PT (H) cells were photographed using an inverted Zeiss microscope at 200×. Bar, 10 μm.

morphologies of breast cancer cells expressing the various members of the cadherin family. MCF-7 cells expressed E-cadherin, had low invasion rates, and presented an epithelial-like morphology. BT-20 cells expressed both E- and P-cadherin, had low invasion rates, and presented an epithelial-like morphology. In contrast, E-cadherin–negative cell lines did not present an epithelial morphology, but rather appeared as fibroblast-like cells with less obvious cell–cell interactions. Even the SUM149 cell line that expressed a small amount of E-cadherin, along with substantial amounts of P-cadherin, did not have the epithelial appearance typified by the MCF-7 and BT-20 cell lines. SUM1315 cells, which expressed P-cadherin, along with a small amount of cadherin-11, also had a fibroblastic appearance with minimal cell–cell interactions. However, these fibroblastic, N-cadherin–negative cell lines had low motility and invasion rates (Table I and Fig. 3). The N-cadherin–expressing cell lines all displayed a fibroblastic phenotype, as typified by MDA-MB-435, MDA-MB-436, and SUM159 (Fig. 2). Cell lines that did not express any cadherin, as typified by SKBr3, displayed a fibroblastic phenotype much like the N-cadherin–positive cells, however, they were less adhesive to the substratum than were cadherin-expressing cells. In addition, they tended to float in the medium upon reaching confluency and when undergoing mitosis.

A Role for N-Cadherin in Cell Motility

In this study, we hypothesized that the invasive behavior

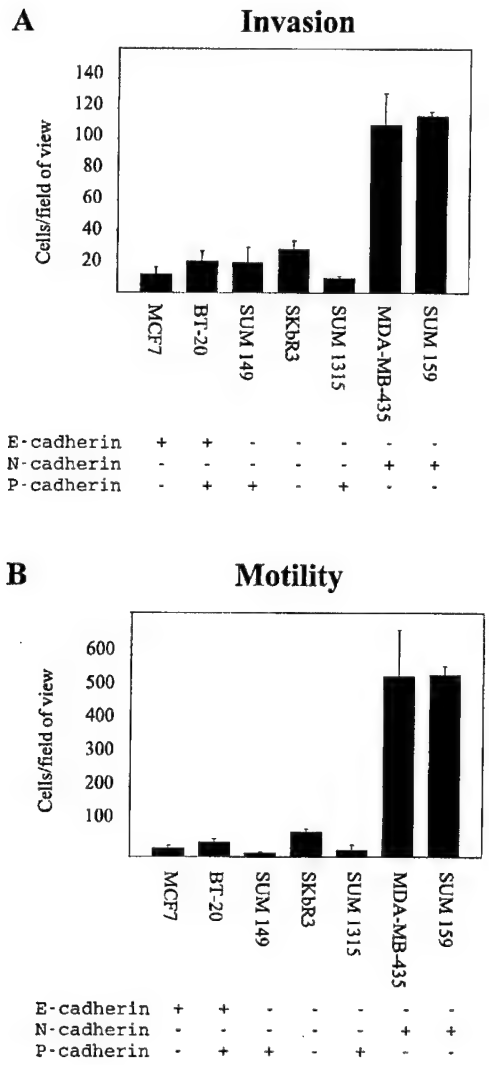

Figure 3. N-cadherin expression correlates with increased invasiveness and motility in breast carcinoma cell lines. Cells were plated on Matrigel-coated or noncoated membranes for invasion assays or motility assays, respectively. The cells were incubated for 24 h, and those that did not migrate through the pores in the membrane were removed by scraping the membrane with a cotton swab. The remaining cells were stained, and the number transversing the membrane was determined by averaging ten random fields of view at 100×. The data are expressed as the number of cells/field of view and is the average of three independent experiments. Error bars indicate SD of the average.

of some breast cancer cell lines may be due to expression of N-cadherin, rather than to lack of expression of E-cadherin. To test this hypothesis, we performed invasion assays on Matrigel-coated membranes and motility assays on uncoated membranes. Fig. 3 presents data from representative cell lines. The N-cadherin–expressing cell lines, SUM159 and MDA-MB-435, were substantially more invasive and more motile than the E-cadherin–expressing line (MCF-7), the E/P-cadherin–expressing cell lines (BT-20 and SUM149), and the P-cadherin–expressing line (SUM 1315). The cell line that did not express any cadherins, SKBr3, was no more motile nor invasive than were the E-cadherin–expressing cell lines BT-20, MCF-7, and SUM 149. Together, these data suggest that, in these cells, N-cadherin acts to promote motility and invasion, rather than E-cadherin acting to suppress these activities.

Since the cell lines in this study were derived from separate tumors and, thus, are likely to be descendents of different cell types, we sought to manipulate expression of specific cadherins in representative cell lines to determine if the invasive phenotype was due to N-cadherin or to other cellular aspects. We chose two cell lines for these studies: BT-20, which expresses E- and P-cadherin and has a low rate of invasion, and MDA-MB-435, which expresses N-cadherin and is highly invasive. When BT-20 cells were transfected with N-cadherin (BT-20N), they expressed levels of N-cadherin that were comparable to MDA-MB-435; however, they did not undergo a morphological change (compare Fig. 2 B with Fig. 4 A), nor did they downregulate the expression of E-cadherin to any significant level. Fig. 4, B and C, show that E- and N-cadherin colocalized at cell–cell borders, suggesting that both cadherins are active at the cell surface. When equal amounts of protein from extracts of BT-20 and BT-20N cells were resolved by SDS-PAGE and immunoblotted for cadherin expression, it could be seen that the BT-20N cells slightly downregulated E-cadherin, that the two cell lines expressed equal levels of P-cadherin, and that the BT-20N cells expressed levels of N-cadherin that were comparable to the invasive N-cadherin–expressing cells depicted in Fig. 1. In addition, β-catenin coimmunoprecipitated equally well with either E- or N-cadherin in these cells (Fig. 4 E). BT-20 cells were unusual in that they expressed high levels of both E- and N-cadherin and, thus, were an ideal cell line in which to test the hypothesis that it is the expression of N-cadherin, not the lack of E-cadherin, that promotes cell motility and invasion in some breast cancer cells. As predicted, motility and invasion rates for BT-20N were five- to eightfold higher than the rates for nontransfected BT-20 cells (Fig. 5). Although BT-20N cells were not as motile as the N-cadherin–expressing MDA-MB-435 cells (Fig. 5 B), they were almost as invasive (Fig. 5 A).

E-Cadherin Does Not Suppress Motility in N-Cadherin–expressing MDA-MB-435 Cells

Since the BT-20N cells expressed high levels of E-cadherin, and were highly motile and invasive, we had good evidence that E-cadherin did not inhibit invasion in these cells and, thus, does not act as an invasion suppressor in all breast cancer cells. However, to further test this idea, we transfected N-cadherin–expressing MDA-MB-435 cells with E-cadherin (MDA-MB-435E) to see if E-cadherin would decrease the invasive nature of these cells. In this experiment, we sought to obtain clones that expressed high levels of E-cadherin, but still retained a significant level of N-cadherin. Fig. 6 D shows the levels of expression of E- and N-cadherin in several clones. Clone 2 was chosen for subsequent studies because it expressed the highest level of E-cadherin and, in addition, showed a two- to threefold reduction in N-cadherin expression, compared

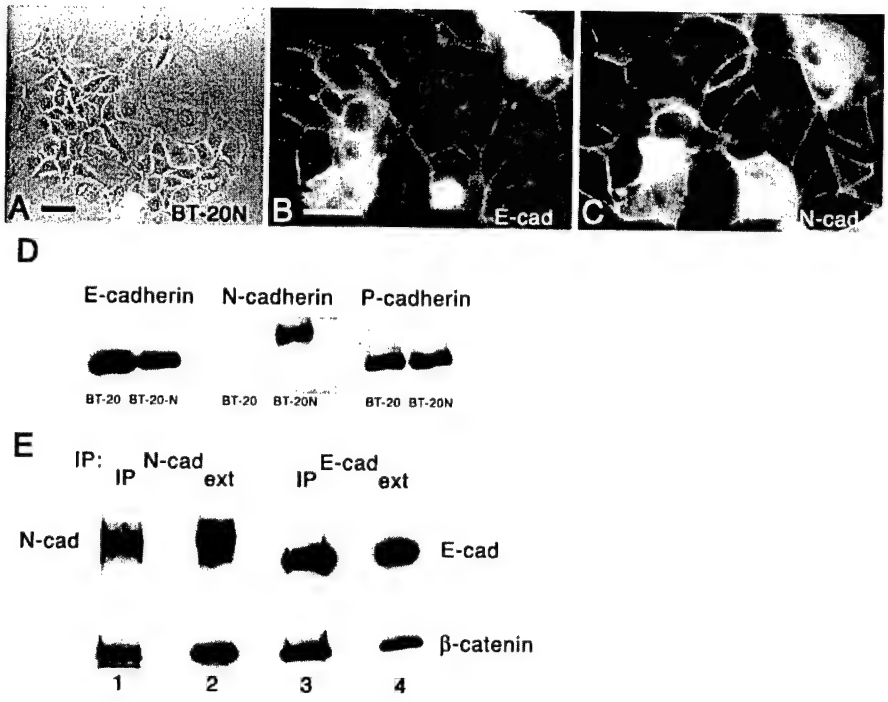

Figure 4. Expression of N-cadherin by BT-20 cells. BT-20 cells were transfected with N-cadherin (BT-20N) and expression induced with dexamethasone. A, Phase-microscopy of living BT-20N cells. Bar, 10 μm. B and C, Cells were grown on glass coverslips and processed for coimmunofluorescence localization with antibodies against E-cadherin (Jelly; B) and N-cadherin (C). D, BT-20 and BT-20N cells were extracted with NP-40 and 20 μg protein from each extract was resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted for E-cadherin (HECD1), N-cadherin, or P-cadherin. E, Extracts of BT-20N cells were immunoprecipitated with antibodies against N-cadherin or E-cadherin (HECD1). The immunoprecipitation reactions, as well as cell extracts, were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted for N-cadherin and β-catenin (lanes 1 and 2) or E-cadherin (HECD1) and β-catenin (lanes 3 and 4).

with the parental cells. Although these cells expressed very high levels of E-cadherin, they did not display a typical epithelial morphology, and closely resembled the parent cell line (compare Figs. 6 A with 2 F). Both E- and N-cadherin were localized to regions of cell–cell contact (Fig. 6, B and C). When the MDA-MB-435E cells were tested for motility and invasion, they were not significantly different from the parental MDA-MB-435 cells (Fig. 5), even though β-catenin was associated with the transfected E-cadherin, as well as the endogenous N-cadherin (Fig. 6 E).

BT-20N Cells Effectively Segregate from HT1080 Fibroblasts

Hazan et al. (1997) suggested that N-cadherin–expressing breast cancer cells invade the stroma because they associate with the N-cadherin–expressing stromal cells. In our studies, we employed an in vitro invasion assay in which the cells invade an extracellular matrix that does not include any stromal cells. Thus, we can make the important statement that, in our studies, N-cadherin actively promotes invasion and motility. In Hazan et al. (1997), the investigators showed that N-cadherin–expressing breast cancer cells coaggregated with N-cadherin–expressing fibroblast-like cells. Since it has been suggested that it is the entire complement of cadherins expressed by a cell that determines its ability to associate with other cells, and that even cells expressing different levels of the same cadherin can sort from one another (Steinberg and Takeichi, 1994), we sought to determine if the BT-20N cells that express N-, E-, and P-cadherin would segregate from an N-cadherin–expressing fibroblast cell line, HT1080. Equal numbers of BT-20 cells and HT1080 cells, or BT-20N cells and HT1080 cells, were mixed together and allowed to settle on glass coverslips. They were then prepared for immunofluorescence analysis using antibodies against E- or N-cadherin. In the immunofluorescence analysis of the BT-20/HT1080 cocultures, E-cadherin stained only the BT-20 cells and N-cadherin stained only the HT1080 cells. Fig. 7, A and B, show that these two cell lines effectively segregated from one another as expected. In the immunofluorescence analysis of the BT-20N/HT1080 cocultures, antibodies against E-cadherin stained only the BT-20N cells, whereas antibodies against N-cadherin stained both the BT-20N cells and the HT1080 cells. Fig. 7, C and D, show that the BT-20N cells and the HT1080 cells effectively segregated from one another, even though both cell lines express N-cadherin. Thus, epithelial cells that express N-cadherin along with other cadherins have not necessarily gained the ability to intermix with stromal cells.

Cadherin-11 Promotes Motility in Breast Epithelial Cells

In the course of our studies on breast tumor cell lines, we characterized one atypical line (MDA-MB-231) that did

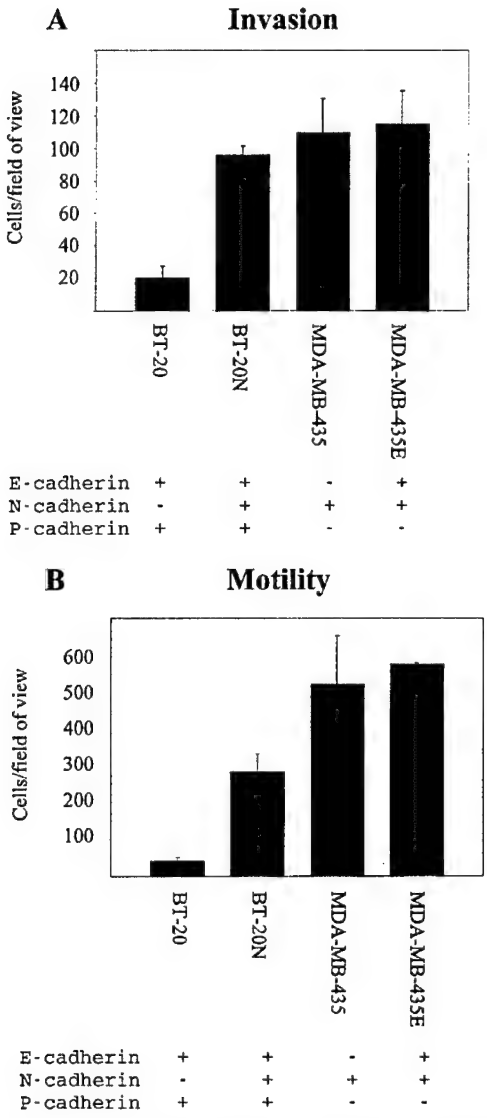

Figure 5. Exogenous expression of N-cadherin by BT-20 cells (BT-20N) increases their invasiveness, whereas exogenous expression of E-cadherin by MDA-MB-435 cells (MDA-MB-435E) does not effect their behavior. Cells were plated on Matrigel-coated or noncoated membranes for invasion assays or motility assays, respectively. The cells were incubated for 24 h, and those that did not migrate through the pores in the membrane were removed by scraping the membrane with a cotton swab. The remaining cells were stained, and the number transversing the membrane was determined by averaging ten random fields of view at 100×. The data are expressed as the number of cells/field of view and is the average of three independent experiments. Error bars indicate SD of the average.

not express E-, P-, or N-cadherin, but nonetheless was invasive (Table I). Since MDA-MB-231 cells expressed significant levels of β-catenin, a protein that is not stable in cadherin-negative cells, we suspected that this cell line expressed another member of the cadherin family of proteins, possibly one that is closely related to N-cadherin. We therefore analyzed RNA from this line with degenerate PCR primers designed to amplify all cadherins and found that it expressed cadherin-11 mRNA. Expression of cadherin-11 protein was confirmed by immunoblotting data with a cadherin-11–specific mAb, in agreement with recent data (Pishvaian et al., 1999). Like N-cadherin, cadherin-11 is expressed by some mesenchymal cells (Simonneau et al., 1995). Interestingly, cadherin-11 is expressed in some epithelial cells of the human placenta, and it has been suggested that cadherin-11 plays a role in mediating trophoblast–endometrium interactions as the cytotrophoblasts invade the uterine wall (MacCalman et al., 1996). Thus, one idea is that cadherin-11 could act in a manner similar to N-cadherin in promoting cell motility and invasion in breast cancer cells. To test this idea, we transfected cadherin-11 into BT-20 cells (BT-20Cad-11 cells). Like the BT-20N cells, BT-20Cad-11 cells retained the morphology of their parent line, even though they expressed high levels of cadherin-11 at cell–cell borders (Fig. 8, A–C). As predicted, cadherin-11–expressing BT-20 cells were more invasive and motile than the parental BT-20 cells (Fig. 8, D and E). Interestingly, the cadherin-11–expressing cells were not as invasive or motile as the N-cadherin–expressing cells. For example, the MDA-MB-231 cells were not as motile as the MDA-MB-435 cells (Figs. 5 and 8). More significantly, the BT-20 cells transfected with cadherin-11 did not become as motile as they did when transfected with N-cadherin. This may be due to differences between the two cadherins, or differences in expression levels of the transfected cadherins. It is reasonable to speculate that the level of expression of the inappropriate cadherin is relevant since the cell line SUM1315 expresses a small amount of cadherin-11, yet is not invasive.

N-Cadherin May Promote Cell Motility through a Fibroblast Growth Factor Receptor Signal Transduction Pathway

The laboratories of Frank Walsh and Patrick Doherty have shown that N-cadherin promotes neurite outgrowth from cerebellar neurons (Williams et al., 1994a). In addition, they showed that N-cadherin–mediated neurite extension was dependent on FGF receptor signaling, but was independent of ligand (Williams et al., 1994b). Walsh and Doherty thus proposed a model whereby the FGF receptor was induced to dimerize in the absence of FGF via interaction with N-cadherin (Doherty and Walsh, 1996). Dimerization of the FGF receptor results in receptor cross phosphorylation that initiates a number of signal transduction pathways. The pathway relevant to N-cadherin–dependent neurite outgrowth involves the generation of arachidonic acid from diacylglycerol, by the action of diacylglycerol lipase. The Walsh and Doherty laboratories showed that the diacylglycerol lipase inhibitor, RHC 80267, prevented neurite extension on N-cadherin–transfected 3T3 cells, thus implicating this type of FGF receptor signaling in N-cadherin–dependent neurite extension (Meiri et al., 1998). We hypothesized that the N-cadherin–mediated cell motility we observed in epithelial cells may also be acting through FGF receptor signaling. To test this hypothesis, we treated MDA-MB-435 cells, BT-20 cells,

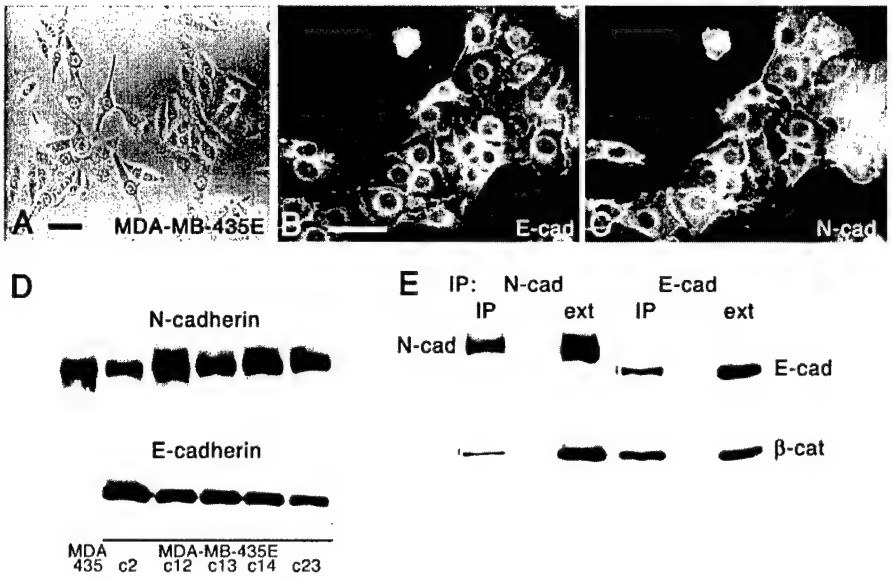

Figure 6. Expression of E-cadherin by MDA-MB-435 cells. MDA-MB-435 cells were transfected with E-cadherin (MDA-MB-435E) and expression was induced with dexamethasone. A, Phase-microscopy of MDA-MB-435E cells. Bar, 10 μm. B and C, Cells were grown on glass coverslips and processed for coimmunofluorescence localization with antibodies against E- (Jelly; B) and N-cadherin (C). D, MDA-MB-435 and several clones of MDA-MB-435E cells were extracted with NP-40 and 20 μg protein from each extract was resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted for E- (HECD1) and N-cadherin. Clone 2 (cl2) expressed the highest level of E-cadherin and was chosen for subsequent studies. E, Extracts of MDA-MB-435 and MDA-MB-435E cells were immunoprecipitated with antibodies against N- or E-cadherin (HECD1). The immunoprecipitation reactions, along with cell extracts, were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted for N-cadherin and β-catenin (lanes 1 and 2), or E-cadherin (HECD1) and β-catenin (lanes 3 and 4).

and BT-20N cells with varying levels of RHC80267 to determine if it would influence the motility of these cells in the transwell assay. RHC80267 inhibited cell motility in both N-cadherin–expressing cell lines in a dose-dependent manner (Fig. 9 A). Importantly, this inhibitor had no effect on the motility of the N-cadherin–negative BT-20 cells. Although these data are consistent with the hypothesis that N-cadherin dependent cell motility is mediated through FGF receptor signaling in a manner similar to N-cadherin–dependent neurite outgrowth, additional experiments must be done to further support this notion. Thus, we are continuing to investigate the mechanism whereby N-cadherin mediates motility in epithelial cells. To determine if cadherin-11 and N-cadherin promote cell motility through a similar pathway, we treated MDA-MB-231 and BT-20cad11 cells with RHC80267, and compared

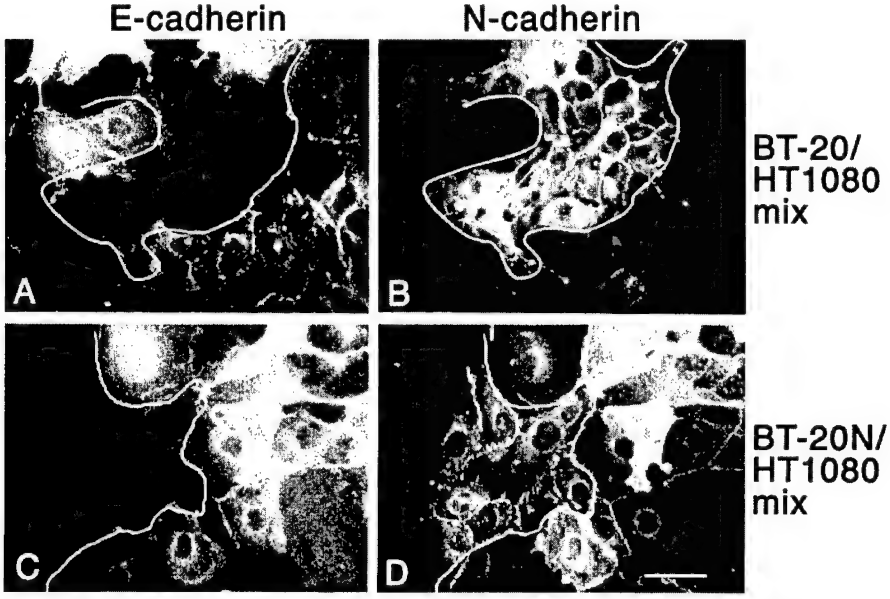

Figure 7. BT-20N cells do not mix with HT1080 cells. 5×10^4 BT-20 or BT-20N cells were mixed with an equal number of HT1080 cells, allowed to settle on coverslips, and processed for immunofluorescence with an mAb against N- (13A9) or E-cadherin (Jelly). A and B are a mix of BT-20 and HT1080 cells stained for E- and N-cadherin, respectively. The encircled cells are a group of E-cadherin–negative, N-cadherin–positive HT1080 cells. C and D are a mix of BT-20N and HT1080 cells stained for E- and N-cadherin, respectively. The encircled cells are a group of E-cadherin–negative, N-cadherin–positive HT1080 cells.

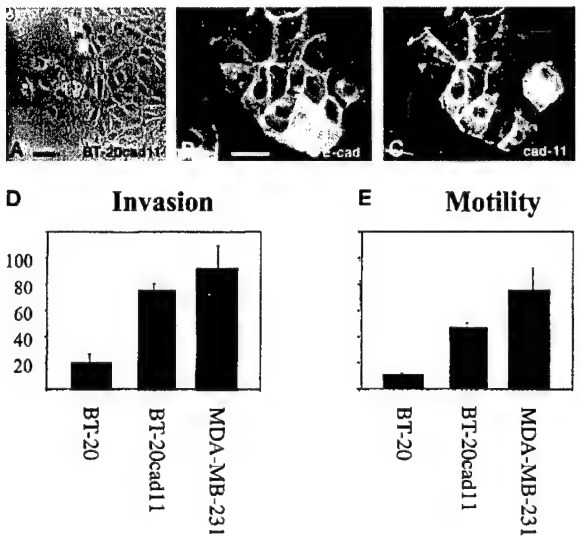

Figure 8. Exogenous expression of cadherin-11 by BT-20 cells (BT-20cad11) increases their invasiveness. BT-20 cells were transfected with cadherin-11 (BT-20cad11) and expression induced with dexamethasone. A, Phase-microscopy of living BT-20cad11 cells. Bars, 10 μm. B and C, Cells were grown on glass coverslips and processed for coimmunofluorescence localization with antibodies against E-cadherin (Jelly; B) and cadherin-11 (C). D and E, Cells were plated on Matrigel-coated or noncoated membranes for invasion assays or motility assays, respectively. The cells were incubated for 24 h, and those that did not migrate through the pores in the membrane were removed by scraping the membrane with a cotton swab. The remaining cells were stained, and the number transversing the membrane was determined by averaging ten random fields of view at 100 ×. The data are expressed as the number of cells/field of view and is the average of three independent experiments. Error bars indicate SD of the average.

motility rates between treated and nontreated cells (Fig. 9 B). The diacylglycerol lipase inhibitor decreased the motility of cadherin-11–expressing cells in a dose-dependent manner. Cadherin-11–expressing cells are less motile than MDA-MB-435, and the inhibitor is less effective in decreasing the motility of the cadherin-11 expressing cells, suggesting there may be some differences in the respective signal transduction pathways, possibly in growth factor receptor levels or isoforms.

Discussion

Previously, our laboratory showed that expression of different cadherin family members by squamous epithelial cells markedly effected morphology (Islam et al., 1996), i.e., when oral squamous epithelial cells expressed N-cadherin, they converted to a fibroblastic phenotype concurrent with decreased cell–cell adhesion. Thus, when we turned our attention to breast cancer cells for the present study, we were interested not only in the expression of various cadherins by these cells, but also in whether these cadherins influenced the morphology of the cells. We were not surprised to find that breast cancer cells endogenously expressing N-cadherin displayed a fibroblastic phenotype with tenuous cell–cell contacts, whereas breast cancer cells endogenously expressing E-cadherin displayed a typical epithelial morphology. We were, however, surprised to find that transfection of N-cadherin into the E-cadherin–expressing BT-20 breast cancer cell line had no effect on morphology, even though it had a dramatic effect on cell behavior. Equally surprising was the fact that forced expression of E-cadherin had no effect on the morphology of the fibroblastic N-cadherin–expressing MDA-MB-435 cells. Thus, the breast cancer cell lines examined in this study behaved very differently from the oral squamous epithelial lines that we characterized previously. Interestingly, the oral squamous epithelial cells downregulated E-cadherin when they were forced to express N-cadherin, suggesting an inverse relationship between these cadherins. In contrast, the breast cancer cells continued to express their endogenous cadherin(s) when transfected with a different cadherin. The continued expression of endogenous cadherin may account for the lack of morphological change in the transfectants. Thus, the breast cancer cells differ from the oral squamous epithelial cells in two very important ways: first, the oral squamous epithelial cells appear to coregulate cadherins in an inverse manner, whereas these cadherins are independently regulated in breast cancer cells; and second, expression of E-cadherin by the oral squamous epithelial cells is sufficient for epithelial morphology, whereas epithelial morphology in the breast cancer cells appears to depend on other factors, in addition to E-cadherin.

In the present study, we have demonstrated that N-cadherin (or cadherin-11) expression in human breast carcinoma cells promotes an invasive phenotype. By transfecting the BT-20 cells with these nonepithelial cadherins, we have provided evidence for a direct role of these cadherins in cell motility and invasion. Previous studies have correlated the expression of N-cadherin or cadherin-11 with invasion in breast cancer cells. However, in this study, we took the important next step and used transfection studies to show that a cell line that has a low invasion rate could be converted to a highly invasive cell by expression of N-cadherin or cadherin-11. The BT-20 breast cancer cell line provided an important tool for these studies since they did not downregulate E-cadherin when forced to express N-cadherin. Thus, we can conclude that, even in cells expressing high levels of E-cadherin, N-cadherin (or cadherin-11) can promote motility, suggesting that, in this regard, both N-cadherin and cadherin-11 are dominant over E-cadherin. A study by Sommers et al. (1994) supports this idea. These authors showed that transfection of E-cadherin into the E-cadherin–negative breast cancer cell lines, BT549 and HS578, did not decrease the invasive capacity of these cells. These authors suggested that the transfected E-cadherin was not functional; however, these authors were unaware of the fact that the BT549 and HS578 cell lines express N-cadherin.

A previous study using MDA-MB-435 cells showed that transfection of E-cadherin into these cells reduced their capacity to form tumors when injected into the foot pads of nude mice (Meiners et al., 1998). In contrast to our study, these authors showed that E-cadherin–transfected clones of MDA-MB-435 cells underwent a morphological

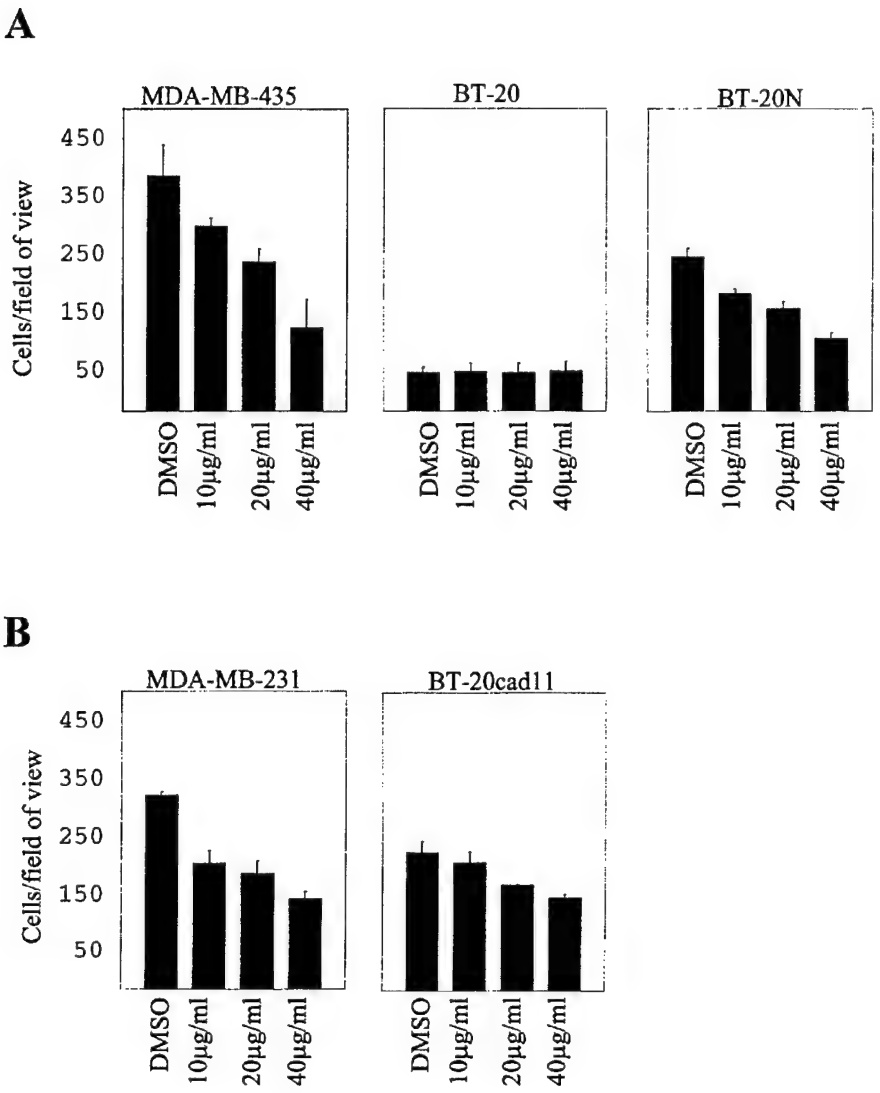

Figure 9. The diacylglycerol lipase inhibitor RHC80267 decreases motility of N-cadherin– and cadherin-11–expressing cells. Cells were plated on noncoated membranes for motility assays. The cells were incubated for 24 h in the presence of RHC80267 at varying concentrations, and those that did not migrate through the pores in the membrane were removed by scraping the membrane with a cotton swab. The remaining cells were stained, and the number transversing the membrane was determined by averaging ten random fields of view at 100×. The data are expressed as the number of cells/field of view and is the average of three (A) or two (B) independent experiments. Error bars indicate SD of the average.

change upon E-cadherin expression. In addition, they showed that E-cadherin–transfected clones were less tumorigenic in their assay than the parental cells. One difference in the study of Meiners et al. (1998) and ours is that they did not assay for N-cadherin expression in their E-cadherin–positive clones of MDA-MB-435 transfectants. Our study clearly demonstrates that N-cadherin influences the behavior of the cells, and that cells retaining N-cadherin do not undergo a morphological or behavioral change upon expression of E-cadherin. Thus, one possible explanation for the difference between these two studies is that the cells in the Meiners' study did not express N-cadherin. The point of our study was to determine if N-cadherin was capable of influencing the behavior of epithelial cells, even if they expressed E-cadherin, thus, we were particularly careful to select cell lines that retained N-cadherin expression after transfection with E-cadherin (Fig. 6).

One puzzling aspect of cell lines derived from metastatic tumors is that they often express E-cadherin and appear to be relatively normal epithelial cells. A possibility suggested by the present study is that such cells may have upregulated the expression of N-cadherin during the process of metastasis. Our results suggest that expression of N-cadherin would confer on these cells the capacity to invade, even though they continued to express E-cadherin. In this regard, expression of an inappropriate cadherin like N-cadherin (or other related cadherins) may be a better gauge of the clinical state of a tumor than is decreased expression of E-cadherin.

Some of the E-cadherin–negative breast cancer cells expressed endogenous P-cadherin. These cells had a fibroblastic morphology similar to that of the N-cadherin–expressing cells; however, they were not highly invasive, suggesting that P-cadherin confers upon breast cancer cells characteristics different from those conferred by either E- or N-cadherin. P-cadherin is expressed in the myo-

epithelial cells surrounding the lumenal epithelial cells of the mammary gland. Radice et al. (1997) recently showed that P-cadherin deficient mice develop age-dependent hyperplasia and dysplasia of the mammary epithelium, and suggested that P-cadherin may play a role in maintaining the normal phenotype of breast epithelial cells. One possibility is that the P-cadherin–expressing tumor cells were derived from the myoepithelium, rather than from the true epithelium.

E-cadherin has been termed an invasion suppressor because transfection of this protein into some E-cadherin–negative invasive carcinoma cells resulted in decreased invasive capacity. Our prediction is that at least some of these cell lines cells expressed a cadherin, like N-cadherin or cadherin-11, and overexpression of E-cadherin resulted in downregulation of the endogenous cadherin, as we saw with the oral squamous epithelial cells. Thus, we hypothesize that the invasion suppressor role of E-cadherin arises in part from its ability to decrease the level of N-cadherin in certain, but not all, tumors. In the present study, cell lines that did not express any classical cadherins, as evidenced by lack of β-catenin protein, as well as lack of detectable cadherin, had low invasion rates. Our hypothesis, that loss of E-cadherin alone does not necessarily increase invasive capacity in breast carcinoma cells, is supported by the observation that function-blocking antibodies against E-cadherin did not confer a highly motile, invasive phenotype on MCF-7 cells, a breast cancer cell line that is E-cadherin–positive and N-cadherin–negative (Sommers et al., 1991). The current study suggests that, in some carcinoma cells, expression of N-cadherin, or a similar cadherin such as cadherin-11, may actually be necessary for increased motility and invasion. A recent clinical study suggested that inactivation of E-cadherin is an early event in the progression of lobular breast carcinomas (Vos et al., 1997). We might suggest that a subsequent event would be activation of the expression of an inappropriate cadherin, such as N-cadherin or cadherin-11.

Understanding the mechanism by which N-cadherin promotes motility in epithelial cells is important if we are to develop treatments that will decrease the invasiveness of tumor cells. A number of studies have shown that epithelial cells can be induced to scatter in response to growth factors, such as hepatocyte growth factor and members of the FGF, EGF, and TGF families (Blay and Brown, 1985; Vallés et al., 1990; Behrens et al., 1991; Geimer and Bade, 1991; Gherardi and Stoker, 1991; Rosen et al., 1991; Miettinen et al., 1994; Savagner et al., 1994, 1997). Walsh, Doherty, and coworkers have established, through extensive studies on FGF receptor and cell adhesion molecules, that N-cadherin and the FGF receptor cooperate to induce neurite outgrowth in cerebellar neurons (reviewed in Doherty and Walsh, 1996; Walsh and Doherty, 1997). These authors have proposed a scheme for activation of the kinase activity of the FGF receptor through cis interactions with N-cadherin, via an HAV domain in the FGF receptor and an HAV interaction domain in the fourth extracellular domain of N-cadherin (Doherty and Walsh, 1996). In addition, it has been proposed that the cadherins form lateral dimers in the plane of the membrane (Shapiro et al., 1995; Takeda et al., 1999), which could result in dimerization of the FGF receptor, and subsequent activation of the signal transduction pathway. We based the studies presented herein on the model presented by Walsh and Doherty, and proposed that interaction of N-cadherin with the FGF receptor in N-cadherin–expressing epithelial cells may result in increased motility, similar to that seen by treating epithelial cells with growth factors. To test this hypothesis, we interfered with the N-cadherin–dependent FGF receptor signal transduction pathway proposed by Walsh and Doherty by inhibiting a downstream enzyme, diacylglycerol lipase. We showed that inhibiting diacylglycerol lipase decreased motility of N-cadherin–expressing cells in a dose-dependent manner while having no effect on the motility of N-cadherin–negative cells. Thus, our data strongly support the notion that N-cadherin promotes motility in breast cancer cells by activating growth factor receptor signal transduction pathways. Continued efforts in our laboratory are aimed at further defining the signal transduction pathway(s) that mediate cadherin-dependent motility in epithelial cells.

At first glance, it might seem unlikely that expression of an additional cell adhesion molecule would confer a motile and invasive phenotype upon an epithelial cell. However, motile cells, such as fibroblasts and myoblasts, express N-cadherin (Knudsen et al., 1995; Huttenlocher et al., 1998) and a switch from E- to N-cadherin occurs in the chick embryo when epiblast cells ingress through the primitive streak to form the mesoderm (Edelman et al., 1983; Hatta and Takeichi, 1986). Another interesting cadherin switch occurs during establishment of the human placenta, where fetal cytotrophoblast cells invade the vasculature of the uterus. During this invasive process, the cytotrophoblast cells downregulate the expression of E-cadherin and upregulate vascular/endothelial (VE) cadherin (Zhou et al., 1997). Thus, it is feasible to suggest that increased expression of a nonepithelial cell cadherin, such as N-cadherin, could increase the invasive potential of tumor cells. Ongoing studies in our laboratory are designed to determine how N-cadherin differs from E-cadherin in its ability to induce cell motility. We hypothesize that E-cadherin does not have the ability to interact with the relevant growth factor receptors, and we are preparing chimeric molecules between E- and N-cadherin to test this hypothesis.

An important message from the present studies is that cadherins may not function identically in different cell types. The fact that cadherins may act differently in different cell types is particularly evident when comparing the current study with earlier studies showing that mouse L cells or S180 fibroblasts attained an epithelial morphology when transfected with either E- or N-cadherin (Nagafuchi et al., 1987; Hatta et al., 1988; Matsuzaki et al., 1990). It will be important in future studies to consider the cellular makeup, as well as the complement of cadherin family members, when interpreting data on cellular morphology and behavior.

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N-Cadherin Extracellular Repeat 4 Mediates Epithelial to Mesenchymal Transition and Increased Motility

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Abstract. E- and N-cadherin are members of the classical cadherin family of proteins. E-cadherin plays an important role in maintaining the normal phenotype of epithelial cells. Previous studies from our laboratory and other laboratories have shown that inappropriate expression of N-cadherin by tumor cells derived from epithelial tissue results in conversion of the cell to a more fibroblast-like cell, with increased motility and invasion. Our present study was designed to determine which domains of N-cadherin make it different from E-cadherin, with respect to altering cellular behavior, such as which domains are responsible for the epithelial to mesenchymal transition and increased cell motility and invasion. To address this question, we constructed chimeric cadherins comprised of selected domains of E- and N-cadherin. The chimeras were transfected into epithelial cells to determine their effect on cell morphology and cellular behavior. We found that a 69–amino acid portion of EC-4 of N-cadherin was necessary and sufficient to promote both an epithelial to mesenchymal transition in squamous epithelial cells and increased cell motility. Here, we show that different cadherin family members promote different cellular behaviors. In addition, we identify a novel activity that can be ascribed to the extracellular domain of N-cadherin.

Key words: N-cadherin • E-cadherin • cancer • motility • invasion

Introduction

Cadherins comprise a family of calcium-dependent cell–cell adhesion proteins that play important roles in embryonic development and maintenance of normal tissue architecture. As the transmembrane component of cellular junctions, the cadherins are composed of three segments: an extracellular domain comprised of five homologous repeats that mediate adhesion, a single pass transmembrane domain, and a conserved cytoplasmic domain that interacts with catenins to link cadherins to the actin cytoskeleton (for review see Wheelock et al., 1996). The catenins were first identified as proteins that coimmunoprecipitated with cadherins and were termed α-, β-, and γ-catenin, according to their mobility on SDS-PAGE. Either β- or γ-catenin binds directly to the cadherin and α-catenin, whereas α-catenin associates directly and indirectly with actin filaments (Stappert and Kemler, 1994; Knudsen et al., 1995; Rimm et al., 1995; Nieset et al., 1997). The ability of cadherins to simultaneously self-associate and link to the actin cytoskeleton mediates both the cell recognition required for cell sorting and the strong cell–cell adhesion needed to form tissues.

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In addition to their structural role in the adherens junction, catenins are thought to regulate the adhesive activity of cadherins. For example, phosphorylation of β-catenin in Src-transformed cells may contribute to the nonadhesive phenotype of these cells (Matsuyoshi et al., 1992; Hamaguchi et al., 1993). As a signaling molecule, β-catenin plays a critical role in patterning during development and in maintenance of the normal cellular phenotype during tumorigenesis (Cadigan and Nusse, 1997; Miller et al., 1999; Polakis et al., 1999). The signaling functions of β-catenin are due to its interactions with transcription factors of the lymphoid enhancer factor/T cell factor (LEF/TCF) family and with receptor tyrosine kinases. In addition, $p120^{ctn}$, originally identified as a Src substrate and subsequently shown to bind to the cytoplasmic domain of cadherins, has been suggested to play a role in regulating the adhesive activity of cadherins (Reynolds et al., 1994; Daniel and Reynolds, 1995; Shibamoto et al., 1995). $p120^{ctn}$ binds to the juxtamembrane domain of cadherins, a domain that has been implicated in cadherin clustering and cell motility (Chen et al., 1997; Finnemann et al., 1997; Navarro et al., 1998; Yap et al., 1998). It is thought that $p120^{ctn}$ influences the strength of cadherin-mediated adhesion, perhaps by influencing the organization of the actin cytoskeleton (Aono et al., 1999; Ohkubo and Ozawa, 1999;

Thoreson et al., 2000). Thus, various studies have shown that the cytoplasmic domain of cadherins interacts with proteins that likely regulate adhesive function.

The extracellular domain of classic cadherins is involved in interactions that mediate adhesion. The earliest evidence for this came from studies demonstrating that antibodies produced against the extracellular domain of cadherins inhibit cell adhesion. The extracellular domain of cadherins can be divided into "extracellular cadherin structural domains" (EC)[1] each of which consists of ~110 amino acids and contains the conserved motifs LDRE, DXNDN, and DXD (Oda et al., 1994). EC-1 is the most NH_2-terminal domain and is responsible for adhesive activity (for review see Takeichi, 1990). The binding sites for most mAbs that block the adhesive function of E-, P-, and N-cadherin have been mapped to EC-1 (for review see Takeichi, 1990), a domain that contains an HAV tripeptide that has been implicated in adhesion. Synthetic peptides containing an HAV sequence inhibit cadherin-mediated adhesion, mimicking the activity of antibodies directed against EC-1 (Blaschuk et al., 1990). Structural studies have shown that the HAV tripeptide and its surrounding residues mediate self-association by interacting with a separate set of amino acids within EC-1 of the interacting cadherin on the adjacent cell (Shapiro et al., 1995). In addition, mutations in the NH_2 terminus of classical cadherins or deletion of EC-1 results in molecules that do not mediate cell adhesion (Nose et al., 1990; Ozawa et al., 1990; Ozawa and Kemler, 1990; Shan et al., 2000).

It was observed that cells expressing different members of the classical cadherin family segregate from one another when mixed together in culture (for review see Takeichi, 1990). It has been suggested that this preferential binding of cadherins plays an important role in the sorting activities of embryonic cells. Interestingly, the binding specificity of cadherin molecules also maps to EC-1. When the NH_2-terminal regions of E-cadherin were replaced with those of P- or N-cadherin, the chimeric molecules displayed P- or N-cadherin specificity, respectively (Nose et al., 1990; Shan et al., 2000). Thus, EC-1 of the classical cadherins is responsible not only for cadherin binding activity, but also for cadherin specificity.

Various studies have implicated E-cadherin in maintenance of the normal phenotype of epithelial cells (for reviews see El-Bahrawy and Pignatelli, 1998; Behrens, 1999). For example, invasive, fibroblast-like carcinoma cells could be converted to a noninvasive phenotype by transfection with a cDNA encoding E-cadherin (Frixen et al., 1991), and forced expression of E-cadherin in rat astrocytoma cells suppressed motility (Chen et al., 1997). Likewise, transfection of invasive E-cadherin–negative cell lines with E-cadherin resulted in cells that were less invasive in in vitro assays (Frixen et al., 1991; Luo et al., 1999). It has been suggested that, unlike E-cadherin, N-cadherin may promote motility and invasion in carcinoma cells. For example, Hazan et al. (1997) reported that expression of N-cadherin by breast carcinoma cells correlated with invasion and suggested that invasion was potentiated by N-cadherin–mediated interactions between the cancer and stromal cells. Studies from our laboratory suggest that N-cadherin plays a direct role in invasion. Expression of N-cadherin by squamous epithelial cells resulted in a scattered phenotype accompanied by an epithelial to mesenchyme transition. Here, forced expression of N-cadherin in cultured cells resulted in downregulation of the expression of E-cadherin (Islam et al., 1996). Thus, it was difficult to separate the characteristics due to decreased expression of E-cadherin from those due to increased expression of N-cadherin. In a second study, we showed that expression of N-cadherin by BT-20 human breast epithelial cells converted the cells to a motile and invasive phenotype. In this case, increased motility was not accompanied by decreased E-cadherin expression, suggesting that N-cadherin plays a direct role in epithelial cell motility (Nieman et al., 1999a). Hazan et al. (2000) confirmed our results using the MCF7 human breast carcinoma cell line. Importantly, these authors extended their studies to show that N-cadherin expression increased metastasis when the transfected cells were injected into nude mice. Thus, there is evidence that expression of an inappropriate cadherin may alter cellular behavior, suggesting that cadherins function as more than just cell–cell adhesion molecules.

Our study was designed to determine which domains of N-cadherin are responsible for both the epithelial to mesenchymal transition that we have seen in squamous epithelial cells and the increased motility seen in breast cancer cells. To address this question, we made use of chimeric cadherins constructed between N-cadherin and E-cadherin. The chimeras were transfected into the SCC1 oral squamous epithelial cell line, to determine their effect on cell morphology, and into the BT20 breast cancer cell line, to investigate influences on cell behavior. We found that a 69–amino acid portion of EC-4 of N-cadherin was both necessary and sufficient to promote motility. This study makes two important points: (a) it shows that cadherins promote differential cellular behavior and (b) it identifies a novel activity that maps to the extracellular domain of N-cadherin.

Materials and Methods

Antibodies and Reagents

Mouse mAbs against the cytoplasmic domain of human N-cadherin (13A9), α-catenin (1G5), and β-catenin (6E3) have been described previously (Johnson et al., 1993; Knudsen et al., 1995). Mouse mAbs against the extracellular amino acids 92–593 of human N-cadherin (8C11) and the cytoplasmic domain of human E-cadherin (4A2) were prepared as described previously (Johnson et al., 1993). Mouse mAb against the myc-epitope (9E10.2) was a gift from Dr. K. Green (Northwestern University, Chicago, IL). All reagents were from Sigma-Aldrich, unless otherwise indicated.

Cell Culture

The human squamous carcinoma cell line UM-SCC-1 (SCC-1) and the human breast cancer cell line BT20 were maintained in MEM 10% FBS (Hyclone Laboratories). A cadherin-negative derivative of A431 called A431D, which was described previously (Lewis et al., 1997), was maintained in DME 10% FBS.

Molecular Constructs

Human N-cadherin (sequence data available from GenBank/EMBL/DDBJ under accession no. S42303) (a gift of Dr. A. Ben Ze'ev, Weizmann Institute, Rehovot, Israel) and human E-cadherin (sequence data avail-

[1]*Abbreviations used in this paper:* EC, extracellular cadherin structural domains; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor.

Table I.

Chimera		Junction	Details
E/N		LLFL/KRRD	Joins E-cad 731 to N-cad 747
N/E and N/E myc		VVWM/RRRA	Joins N-cad 746 to E-cad 732
N/E5a myc		AGPF/TAEL	Joins N-cad 637 to E-cad 627
N/E5 myc		DNAP/IPEP	Joins N-cad 603 to E-cad 594
N/E4 myc		NIRY/RIWR	Joins N-cad 534 to E-cad 524
N/E3 myc		NAVY/TILN	Joins N-cad 420 to E-cad 414
N/E2 myc		MLRY/TILS	Joins N-cad 306 to E-cad 303
E/N/E myc	5′	KITY/TKLS	Joins E-cad 523 to N-cad 535
	3′	DNAP/IPEP	Joins N-cad 603 to E-cad 594
N/E/N myc	5′	NIRY/RIWR	Joins N-cad 534 to E-cad 524
	3′	DNAP/QVLP	Joins E-cad 593 to N-cad 604

In our N-cadherin cDNA, there is an additional leucine (CTG) after amino acid 11. Thus, the entire open reading frame is 906 codons. The numbers in the table reflect this change to S42303. The E-cadherin cDNA has an open reading frame of 882 codons.

able from GenBank/EMBL/DDBJ under accession no. Z13009) (Lewis et al., 1997) were used for construction of chimeric cadherins using recombinant PCR (Higuchi et al., 1988). In each case, the recombinant PCR product was subcloned and representatives were sequenced until one was identified that encoded the complete, correct amino acid sequence. Each full-length construct was assembled by joining restriction fragments from the correct recombinant PCR product and the cDNA clones. The full-length construct was moved into pLKneo (Hirt et al., 1992) or a derivative for transfection into cells. Amino acid sequences across the chimeric junctions are given in Table I. Brief descriptions of the constructions are given below; complete details are available upon request.

The E/N-chimera has the extracellular and transmembrane domains of E-cadherin and the cytoplasmic domain of N-cadherin, whereas the N/E-chimera has the extracellular and transmembrane domains of N-cadherin and the cytoplasmic domain of E-cadherin. To construct the E/N-chimera, recombinant PCR was used to generate a chimeric cDNA encoding a portion of E-cadherin's extracellular domain, including the unique Bsu36I site, plus its transmembrane domain and N-cadherin's entire cytoplasmic domain. To complete the full-length E/N-chimera, a 5′ E-cadherin cDNA fragment was ligated to the recombinant PCR product at the Bsu36I site. A similar strategy was employed to form the N/E-chimera, except the unique BglII site located in the N-cadherin sequence was used to join the 5′ N-cadherin cDNA fragment to the recombinant PCR product. The full-length chimeras were inserted into pLKneo for transfection.

To make the N/E-myc construct, the cytoplasmic domain of E-cadherin, including the unique SmaI site, was amplified such that the stop codon was replaced with a restriction site. The PCR product was inserted into a modified pSPUTK (Falcone and Andrews, 1991) to add a COOH-terminal 2X-myc tag (Nieman et al., 1999a). A 5′ restriction fragment from the N/E-chimera was ligated to the above construct at the SmaI site to make the full-length N/E-myc cDNA. To make N/E5a-myc, N/E5-myc, and N/E4-myc, recombinant PCR fragments were used to replace portions of the N/E-myc construct by using convenient restriction sites. To make N/E3-myc, a recombinant PCR fragment was used to replace a portion of the N/E4-myc construct. In a similar fashion, the N/E2-myc construct was made by replacing a portion of the N/E3-myc construct with a recombinant PCR fragment. Each of these full-length cadherins was then inserted into pLKpac (Islam et al., 1996) for transfection.

The E/N/E-myc chimera was generated by substituting nucleotides encoding N-cadherin amino acids 535–603 for the corresponding E-cadherin sequence. Recombinant PCR was performed to create the 5′ junction between E- and N-cadherin. The product of this reaction was used in a second recombinant PCR step to create the 3′ junction between N- and E-cadherin. The resulting PCR product was used to replace a portion of E-cadherin–2X-myc (Nieman et al., 1999b). The N/E/N-myc construct was prepared similarly. In this case, the final PCR product was used to replace a portion of the N-cadherin sequence in an N-cadherin–2X-myc construct. The chimeras were inserted into pLKpac for transfection.

Transfections

SCC1 and A431D cells were transfected, using calcium phosphate and BT20, by electroporation, as previously described (Nieman et al., 1999a). Stable clones were selected by growth in puromycin (1 μg/ml) or G418 (1 mg/ml). Clones were screened for transgene expression by immunoblot analysis. Clones that showed homogenous expression by immunofluorescence were selected. For morphological studies, at least three clones from each transfection were examined.

Microscopy

Cells were grown on glass coverslips, fixed with Histochoice (Amresco), blocked using PBS 10% goat serum, and stained with primary antibodies for 1 h, followed by treatment with a secondary antibody (Jackson ImmunoResearch Laboratories). Photos were taken with a ZEISS Axiophot microscope (ZEISS) equipped with a SPOT CCD camera (Spot Diagnostic).

Cell Fractionation and Protein Assays

Confluent monolayers were washed with PBS and extracted on ice with TNE buffer (10 mM Tris-acetate, pH 8.0, 0.5% NP-40, 1 mM EDTA, 2 mM PMSF). Extracts were mixed at 4°C for 30 min and centrifuged at 15,000 rpm for 15 min. Protein determinations were done using a Bio-Rad kit (Bio-Rad Laboratories).

Immunoprecipitations, Electrophoresis, and Immunoblot Analysis

A 300-μl aliquot of cell extract was incubated with 300 μl hybridoma supernatant for 30 min at 4°C. Protein A beads were added, and the incubation was continued for 30 min. Immune complexes were washed with TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Tween 20) 5× at 4°C. Pellets were resolved by SDS-PAGE and immunoblotted as described previously (Johnson et al., 1993).

Aggregation Assays

Aggregation assays were done as described by Redfield et al. (1997), with minor modifications. In brief, cells were trypsinized and resuspended at 2.5×10^5 cells/ml in the appropriate medium containing 10% FBS. 20-μl drops of medium, containing 5,000 cells/drop, were pipetted onto the inner surface of the lid of a petri dish. The lid was then placed on the petri dish so that the drops were hanging from the lid with the cells suspended within them. To eliminate evaporation, 10 ml serum-free culture medium was placed in the bottom of the petri dish. After 24 h at 37°C, the lid of the petri dish was inverted and photographed using a ZEISS inverted tissue culture microscope at 100× magnification.

Motility Assays

For motility assays, 5×10^5 cells were plated in the top chamber of non-coated polyethylene teraphthalate membranes (six-well insert, pore size 8 mm) (Becton Dickinson). 3T3-conditioned medium was used as a chemoattractant in the lower chamber. The cells were incubated for 24 h, and the cells that did not migrate through the pores in the membrane were removed by scraping the membrane with a cotton swab. Cells transversing the membrane were stained with Diff-Quick (Dade). Cells in 10 random fields of view at 100× magnification were counted and expressed as the average number of cells/field of view. Three independent experiments were done in each case. The data was represented as the average of the three independent experiments with the standard deviation of the average indicated. When cells were induced with dexamethasone to express a transgene, the control cells were treated with the same level of dexamethasone.

Antibody Blocking Experiments

Ascites fluid generated from the 8C11 mAb or control ascites was diluted in culture medium. Cells were plated on membranes for motility assays, as described above, except that the cells were plated in medium-containing ascites fluid. After 24 h, the number of cells traversing the membrane was determined.

Results

Previous studies from our laboratory showed that expression of N-cadherin by squamous epithelial cells or breast cancer cells altered cellular behavior. In oral squamous epithelial cells, expression of N-cadherin produced a scattered phenotype with an epithelial to mesenchymal transi-

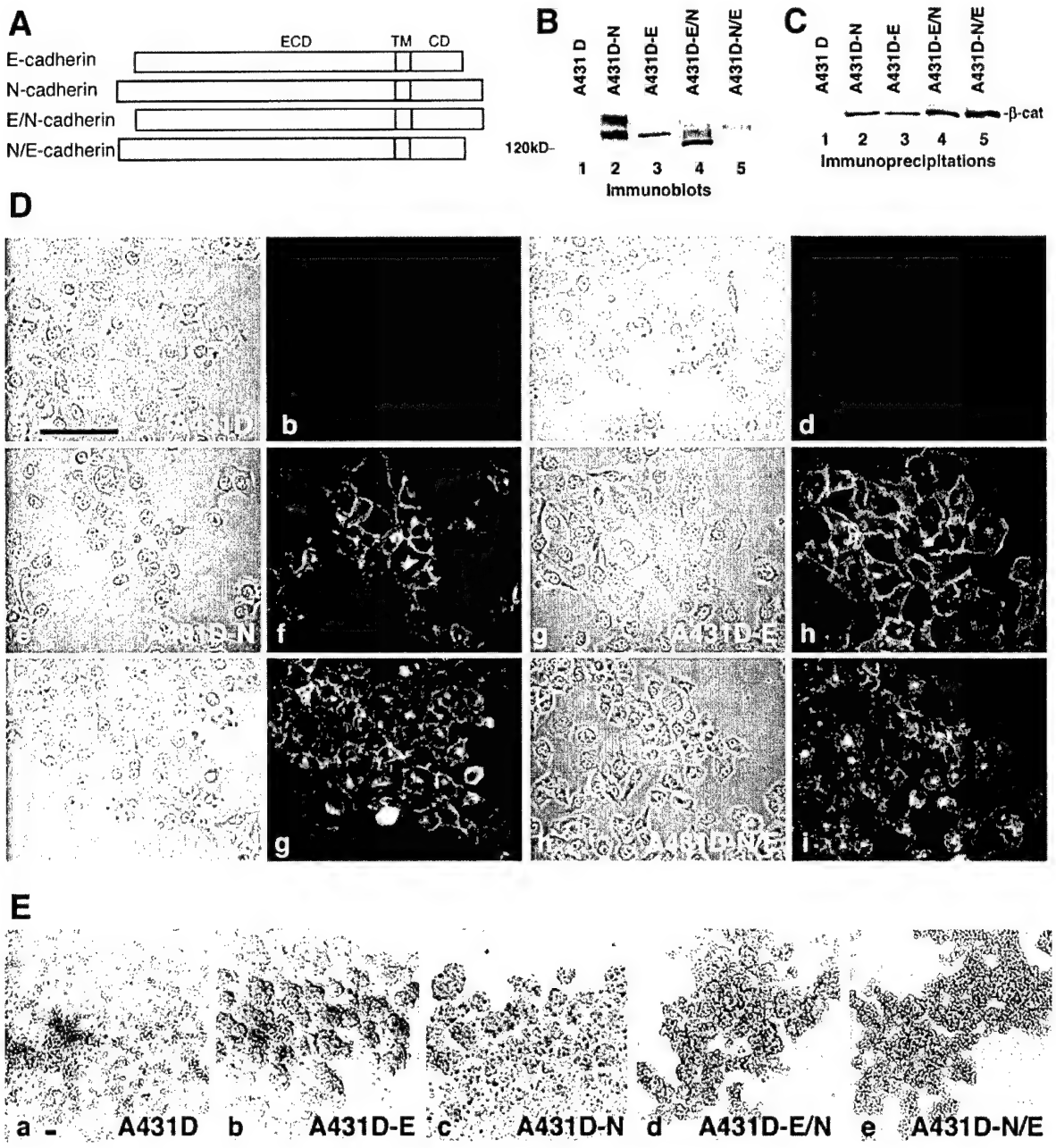

Figure 1. Expression of E/N- and N/E-cadherin in A431D cells. (A) Chimeric cadherins consisting of the extracellular and transmembrane domains of E-cadherin (white) and the cytoplasmic domain of N-cadherin (gray) or consisting of the extracellular and transmembrane domains of N-cadherin and the cytoplasmic domain of E-cadherin were cloned into pLKneo2. (B) A431D cells were transfected with N-cadherin, E-cadherin, E/N-cadherin, or N/E-cadherin and examined for transgene expression by immunoblotting with antibodies against the cytoplasmic domain of N-cadherin (lanes 1, 2, and 4), the extracellular domain of N-cadherin (lane 5), or the extracellular domain of E-cadherin (lane 3). Note, in some cases, we observed various processing variants when transfected cadherins were overexpressed in cells. (C) Extracts were immunoprecipitated, resolved by SDS-PAGE, and immunoblotted for β-catenin. (D) Untransfected A431D cells (a–d) or A431D cells expressing N-cadherin (e and f), E-cadherin (g and h), E/N-cadherin (f and g), or N/E-cadherin (h and i) were processed for immunofluorescence microscopy using the appropriate cadherin antibody. Corresponding phase and fluorescence micrographs are shown. (E) Untransfected A431 D cells (a) or A431D cells expressing E-cadherin (b), N-cadherin (c), E/N-cadherin (d), or N/E-cadherin (e) were tested for their ability to aggregate in a hanging drop aggregation assay. Bar, 10 μm.

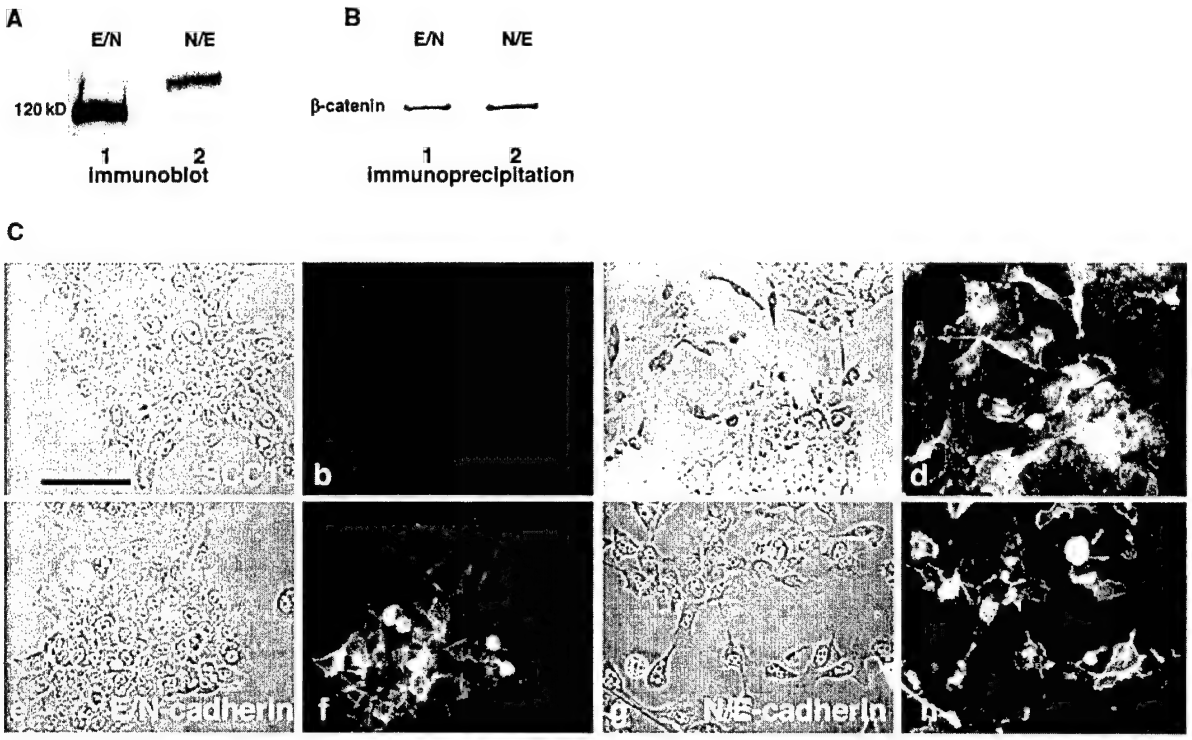

Figure 2. Expression of E/N- and N/E-cadherin in SCC1 cells. (A) SCC1 cells were transfected with E/N- or N/E-cadherin and examined for transgene expression by immunoblotting with antibodies against the cytoplasmic (lane 1) or extracellular (lane 2) domain of N-cadherin. (B) Extracts were immunoprecipitated, resolved by SDS-PAGE, and immunoblotted for β-catenin. (C) Untransfected SCC1 cells (a and b) or SCC1 cells expressing N-cadherin (c and d), E/N-cadherin (e and f), or N/E-cadherin (g and h) were processed for immunofluorescence microscopy using the appropriate cadherin antibody. Corresponding phase and fluorescence micrographs are shown. Bar, 15 μm.

tion (Islam et al., 1996). In breast cancer cells, expression of N-cadherin did not alter the morphology of the cells, but did induce cell motility and invasion (Nieman et al., 1999a). Here, we sought to determine how N-cadherin functioned to alter the phenotype of epithelial cells. We predicted that the cytoplasmic domain of N-cadherin was capable of initiating a signal transduction pathway that resulted in increased cellular motility. To determine if this was the case, we engineered two chimeric cadherins. The first, called E/N-cadherin, consisted of the extracellular and transmembrane domains of E-cadherin joined to the cytoplasmic domain of N-cadherin. The second chimera consisted of the extracellular and transmembrane domains of N-cadherin joined to the cytoplasmic domain of E-cadherin (N/E-cadherin). A schematic of these two chimeric cadherins is presented in Fig. 1 A.

The Extracellular Domain of N-Cadherin Influences Epithelial Cell Behavior

Our goal was to test E/N-cadherin and N/E-cadherin for effects on cellular morphology and behavior using two model systems we had already established. In the first model system, the oral squamous epithelial cell line SCC1 undergoes a significant and readily discernible morphological change from a typical epithelial cell to a fibroblastic cell, when transfected with N-cadherin (Islam et al., 1996). In the second model system, the human breast cancer cell line BT20 changes from a relatively nonmotile to a highly motile cell when transfected with N-cadherin (Nieman et al., 1999a). Interestingly, the BT20 cells do not undergo a morphological change when they are transfected with N-cadherin, suggesting that the effects of N-cadherin differ somewhat between these two different types of epithelial cells. Before testing the effect our chimeric cadherins had on the morphology and behavior of cells, it was important to show that each chimera was a functional adhesion molecule. To determine if the chimeras were functional, we transfected them into the cadherin-negative A431D cell line, which has been previously described by our laboratory (Lewis et al., 1997; Thoreson et al., 2000). Fig. 1 shows that the chimeric cadherins were expressed by the A431D cells at the expected size (Fig. 1 B), that they associated with catenins in an immunoprecipitation assay (Fig. 1 C), that they were located at the cell surface (Fig. 1 D), and that they mediated cell aggregation (Fig. 1 E). These data demonstrate that both E/N-cadherin and N/E-cadherin function as adhesion molecules in a manner similar to E-cadherin or N-cadherin. Surprisingly, the morphology of A431D cells transfected with E-cadherin did not differ significantly from that of A431D cells transfected with N-cadherin. In addition, the morphology of A431D cells transfected with the chimeras was similar to A431D cells transfected with either E-cadherin or N-cadherin.

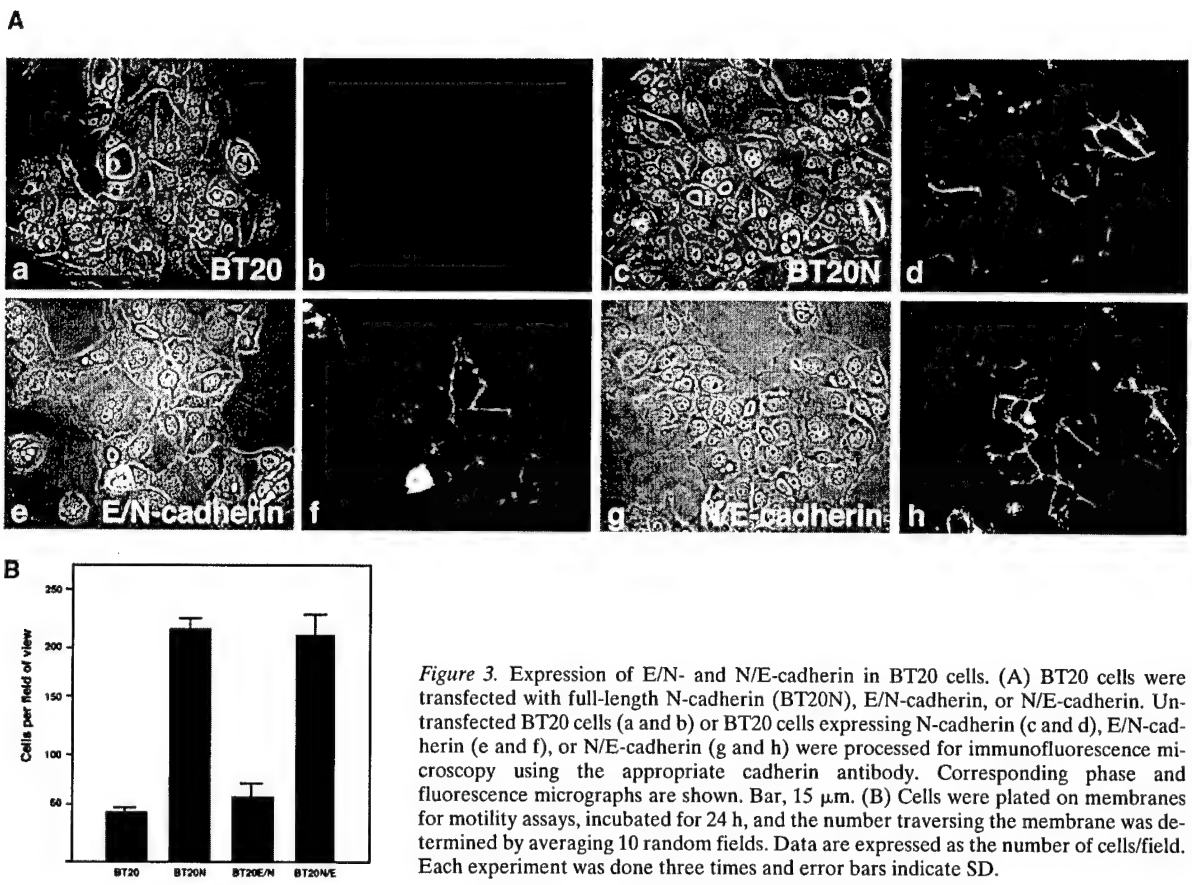

Figure 3. Expression of E/N- and N/E-cadherin in BT20 cells. (A) BT20 cells were transfected with full-length N-cadherin (BT20N), E/N-cadherin, or N/E-cadherin. Untransfected BT20 cells (a and b) or BT20 cells expressing N-cadherin (c and d), E/N-cadherin (e and f), or N/E-cadherin (g and h) were processed for immunofluorescence microscopy using the appropriate cadherin antibody. Corresponding phase and fluorescence micrographs are shown. Bar, 15 μm. (B) Cells were plated on membranes for motility assays, incubated for 24 h, and the number traversing the membrane was determined by averaging 10 random fields. Data are expressed as the number of cells/field. Each experiment was done three times and error bars indicate SD.

E/N-cadherin and N/E-cadherin were transfected into SCC1 cells and analyzed for their ability to induce an epithelial to mesenchymal transition. Each chimera was highly expressed (Fig. 2 A), coimmunoprecipitated with β-catenin (Fig. 2 B), and localized at the cell surface (Fig. 2 C, f and h). To our surprise, the N/E-cadherin (Fig. 2 C, g) produced a change in morphology similar to that seen with intact N-cadherin (Fig. 2 C, c), whereas the E/N-cadherin did not effect the morphology of these cells (Fig. 2 C, e). To determine if the extracellular domain of N-cadherin was also responsible for the change in motility of BT20 cells, we transfected N/E-cadherin and E/N-cadherin into these cells. Fig. 3 A shows that both chimeric cadherins were expressed at the cell surface and that neither chimera produced an effect on the morphology of these cells. This is consistent with our previous studies showing that N-cadherin did not effect the morphology of BT20 cells (Nieman et al., 1999a) (Fig. 3 A, c). Fig. 3 B shows that N/E-cadherin was as efficient as intact N-cadherin at inducing motility in BT20 cells, whereas E/N cadherin did not significantly alter the motile characteristics of BT20 cells. Thus, our hypothesis that the cytoplasmic domain of N-cadherin initiates a signaling pathway, resulting in increased cell motility, was not substantiated. Rather, it appeared that the extracellular domain of N-cadherin was responsible for the epithelial to mesenchymal transition in squamous epithelial cells and increased motility in breast cancer cells. The remainder of this study was aimed at determining which part of the extracellular domain of N-cadherin influenced cellular morphology and behavior.

Extracellular Domain 4 of N-Cadherin Confers a Motile Phenotype on Epithelial Cells

To further investigate the extracellular domain of N-cadherin, we constructed additional chimeric cadherins. We started with N/E-cadherin and moved the boundary between N- and E-cadherin progressively towards the NH_2 terminus (Fig. 4 A). We added a myc tag to the COOH terminus of the chimeras so that we could use the identical antibody to detect each chimera. We also constructed a chimeric N/E-cadherin with a myc tag (N/E-myc) to ensure addition of the tag did not alter the ability of N/E-cadherin to confer a motile phenotype on human epithelial cells. The chimeric cadherin that included approximately one third of EC5 of E-cadherin was designated N/E5a-myc; the chimeric cadherin that included EC5 of E-cadherin was designated N/E5-myc; the chimeric cadherin that included EC5 and most of EC4 of E-cadherin was designated N/E4-myc; the chimeric cadherin that included EC5, EC4, and most of EC3 of E-cadherin was designated N/E3-myc; and the chimeric cadherin that included EC5, EC4, EC3, and most of EC2 of E-cadherin was designated N/E2-myc (Fig. 4 A).

Each chimera was transfected into the cadherin-negative A431D cells to determine if it functioned properly as an adhesion molecule. The N/E-cadherin with a 2X-myc

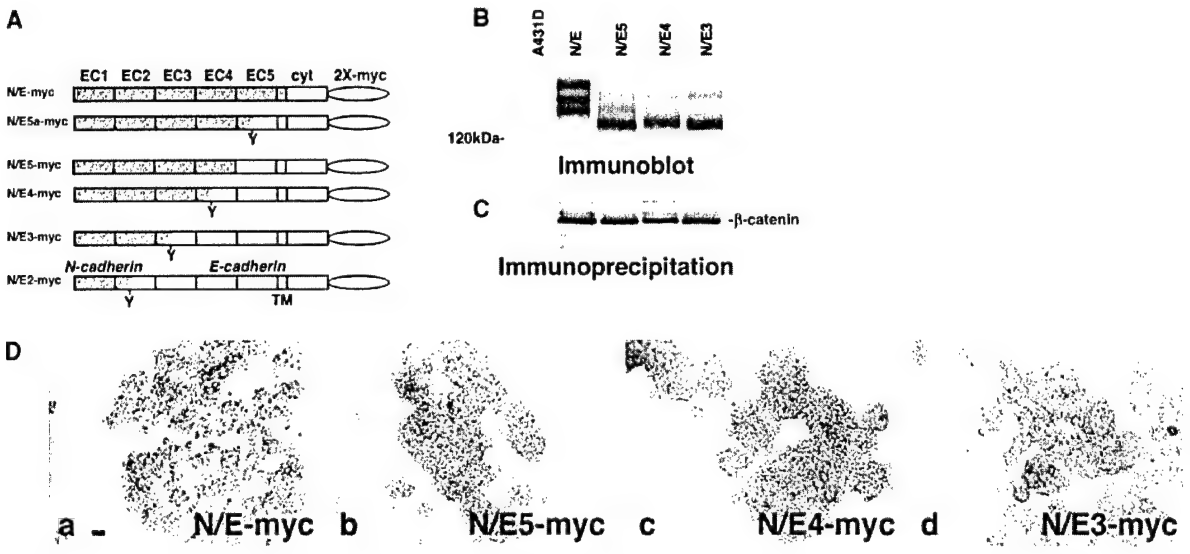

Figure 4. Generation of additional cadherin chimeras. (A) Chimeric cadherins, with a 2X-myc tag at the COOH terminus, consisting of E-cadherin (white) and N-cadherin (gray), were cloned into pLKpac. (B) A431D cells were transfected and examined for transgene expression by immunoblotting with anti-myc. Note, in some cases, we observed various processing variants when transfected cadherins were overexpressed in cells. (C) Extracts were immunoprecipitated using anti-myc, resolved by SDS-PAGE, and immunoblotted for β-catenin. (D) A431D cells expressing N/E-myc-cadherin (a), N/E5-myc-cadherin (b), N/E4-myc-cadherin (c), or N/E3-myc-cadherin (d) were tested for their ability to aggregate in a hanging drop aggregation assay. Bar, 15 μm.

tag (N/E-myc-cadherin) behaved exactly like N/E-cadherin, indicating that the myc tag did not influence the function of the chimeric cadherin. Chimeras N/E-myc, N/E5-myc, N/E4-myc, and N/E3-myc were each expressed at a high level, as indicated by immunoblot analysis using anti-myc antibodies (Fig. 4 B). The proteins were processed to the predicted size, though there was more unprocessed protein than was seen for endogenous cadherins, E/N-cadherin, or N/E-cadherin. Each chimera efficiently associated with β-catenin, as demonstrated by coimmunoprecipitation (Fig. 4 C). In addition, each chimera mediated cell aggregation (Fig. 4 D). Chimeras N/E5a-myc and N/E2-myc were not properly processed or did not mediate adhesion in A431D cells, so we did not use them in assays to map the domain of N-cadherin that functions to induce motility in epithelial cells.

When N/E-myc, N/E5-myc, N/E4-myc, and N/E3-myc chimeric cadherins were transfected into SCC1 cells, they were highly expressed (Fig. 5 A) and coimmunoprecipitated with β-catenin (Fig. 5 B). The N/E-myc and N/E5-myc chimeras produced the same morphological change in SCC1 cells that was seen with N/E-cadherin (Fig. 5 C, a and c). In contrast, the N/E4-myc and N/E3-myc chimeras had no effect on the morphology of SCC1 cells (Fig. 5 C, e and g).

We were equally interested in the ability of these additional chimeric cadherins to influence cellular motility. We typically use the BT20 cells for this assay, since we have established a clear difference between N-cadherin–expressing and –nonexpressing BT20 cells. In addition, we wanted to be sure we were looking at the same phenomenon we had previously published (Nieman et al., 1999a). However, the BT20 cells grow slowly in culture and are difficult to transfect. We have not been successful at establishing BT20 cell lines expressing the additional chimeras. Therefore, we established a motility assay that made use of the already transfected A431D cells. We first showed that A431D cells transfected with N-cadherin were more motile than untransfected A431D cells or A431D cells transfected with E-cadherin (Fig. 5 D). In addition, we showed that A431D cells transfected with E/N-cadherin behave similarly to A431 cells transfected with intact E-cadherin, and A431D cells transfected with N/E-cadherin behave like A431D cells transfected with intact N-cadherin. Thus, we believe we are testing the same N-cadherin–mediated effect on motility whether we use the BT20 system or the A431D system. A431D cells transfected with the N/E5 chimera were as motile as those transfected with full-length N-cadherin or with the N/E chimera, whereas the motility rates of cells transfected with the N/E4 and N/E3 chimeras were similar to the motility rates of cells transfected with E-cadherin or with the E/N chimera. Thus, we determined that the domain of N-cadherin, which is responsible for the epithelial to mesenchymal transition when expressed in squamous epithelial cells, is most likely the same domain that increases cell motility when N-cadherin is expressed in epithelial cells. This domain probably resides in EC4, most likely the region including amino acids 535–603.

Extracellular Domain 4 Is Sufficient to Confer a Motile Phenotype on Epithelial Cells

To confirm that extracellular domain 4 of N-cadherin alone was responsible for altering the behavior of epithelial cells, we constructed two additional chimeric cadherins. The first was E-cadherin, except that amino acids 535–603 of N-cadherin replaced the corresponding portion of E-cadherin and was called E/N/E-cadherin (Fig. 6 A). The second chimera was N-cadherin, except that amino acids 535–603 of N-cadherin were replaced by the corre-

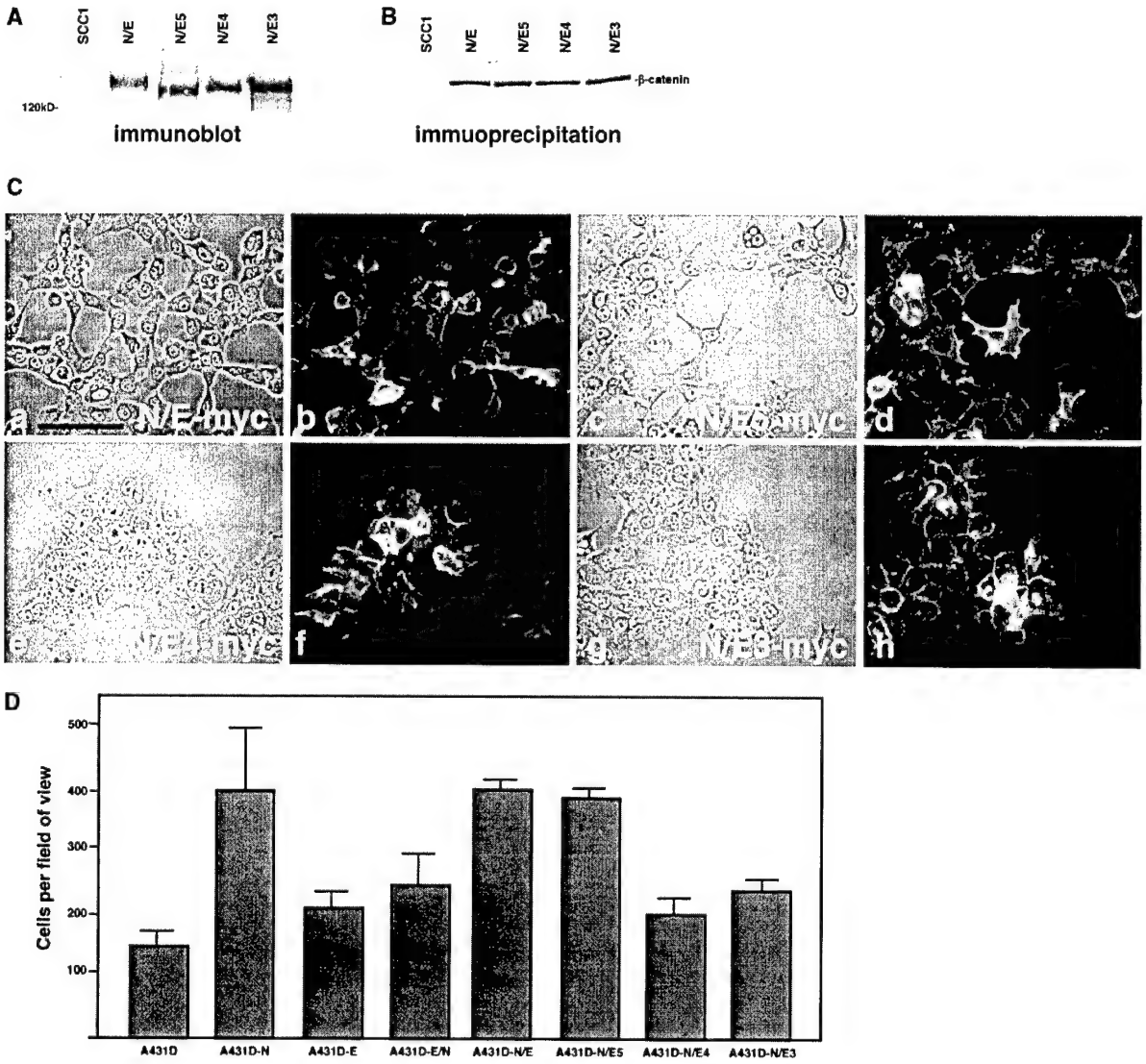

Figure 5. Expression of additional N/E-cadherin chimeras. (A) SCC1 cells were transfected with N/E-myc-cadherin, N/E5-myc-cadherin, N/E4-myc-cadherin, or N/E3-myc-cadherin and examined for transgene expression by immunoblotting with anti-myc. (B) Extracts were immunoprecipitated using anti-myc, resolved by SDS-PAGE, and immunoblotted for β-catenin. (C) SCC1 cells transfected with N/E-myc-cadherin (a and b) N/E5-myc-cadherin (c and d), N/E4-myc-cadherin (e and f), or N/E3-myc-cadherin (g and h) were processed for immunofluorescence microscopy using anti-myc. Corresponding phase and fluorescence micrographs are shown. Bar, 15 μm. (D) A431D cells either nontransfected or transfected with N-cadherin (A431D-N), E-cadherin (A431D-E), E/N-myc-cadherin (A431D-E/N), N/E-myc-cadherin (A431D-N/E), N/E5-myc-cadherin (A431D-N/E5), N/E4-myc-cadherin (A431D-N/E4), or N/E3-myc-cadherin (A431D-N/E3) were plated on membranes for motility assays, incubated for 24 h, and the number traversing the membrane was determined by averaging 10 random fields. Data are expressed as the number of cells/field. Each experiment was done three times and error bars indicate SD.

sponding amino acids of E-cadherin (N/E/N-cadherin). Both chimeras included a 2X-myc tag. When transfected into the cadherin-negative A431D cells, both the E/N/E-cadherin and the N/E/N-cadherin were highly expressed, coimmunoprecipitated with β-catenin (Fig. 6 B), and efficiently mediated cell aggregation (Fig. 6 C). In addition, each chimera was expressed at cell borders in SCC1 cells (Fig. 6 D). The E/N/E chimera produced the epithelial to mesenchymal transition seen with full-length N-cadherin (Fig. 6 D, a), whereas the N/E/N chimera did not (Fig. 6 D, c). When A431D cells were transfected with the E/N/E-cadherin, they showed motility rates similar to that seen when the cells were transfected with full-length N-cadherin. In contrast, the N/E/N transfected cells showed motility rates similar to E-cadherin–transfected cells (Fig. 6 E). Thus, this short 69–amino acid segment of N-cadherin was both necessary and sufficient to cause the morphological and behavioral changes seen in epithelial cells.

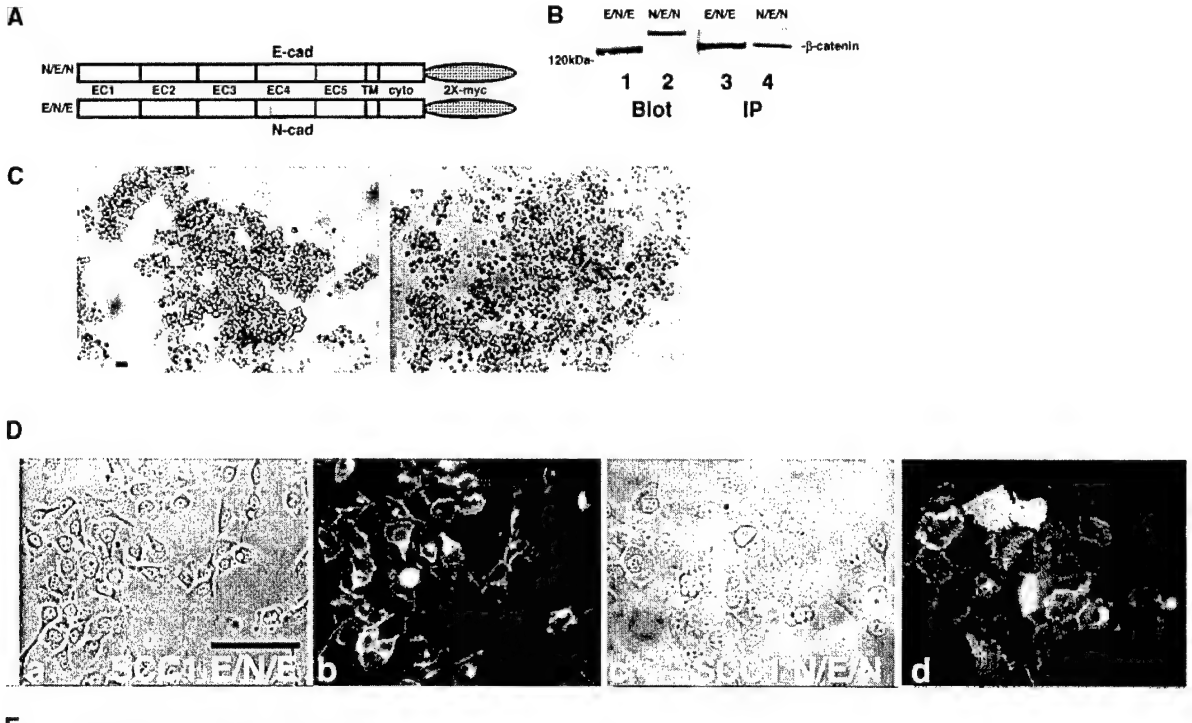

Figure 6. Generation and expression of E/N/E- and N/E/N-cadherins. (A) Chimeric cadherins consisting of E-cadherin (white) and N-cadherin (gray) were constructed and cloned into pLKpac with a 2X-myc tag at the COOH terminus. (B) A431D cells were transfected and examined for transgene expression by immunoblotting with anti-myc (lanes 1 and 2). Extracts were immunoprecipitated using anti-myc, resolved by SDS-PAGE, and immunoblotted for β-catenin (lanes 3 and 4). (C) A431D cells expressing E/N/E-myc-cadherin (a) or N/E/N-myc-cadherin (b) were tested for their ability to aggregate in a hanging drop aggregation assay. (D) SCC1 cells transfected with E/N/E-myc-cadherin (a and b) or N/E/N-myc-cadherin (c and d) were processed for immunofluorescence microscopy using anti-myc. Corresponding phase and fluorescence micrographs are shown. Bar, 15 μm. (E) A431D cells transfected with E-cadherin (A431D-E), N-cadherin (A431D-N), E/N/E-myc-cadherin (E/N/E), or N/E/N-myc-cadherin (N/E/N) were plated on membranes for motility assays, incubated for 24 h, and the number traversing the membrane was determined by averaging 10 random fields. Data are expressed as the number of cells/field. Each experiment was done three times and error bars indicate SD.

Antibodies Directed against the Extracellular Domain of N-Cadherin Inhibit Motility in Epithelial Cells

The domain of classical cadherins that is responsible for cell adhesion resides in EC1. Antibodies directed against EC1 inhibit cadherin-mediated cell–cell interactions. Thus, we sought to determine if the ability of N-cadherin to influence cellular behavior could be inhibited by antibodies that bind to EC4. We immunized mice with the entire extracellular domain of human N-cadherin and chose those antibodies that mapped near EC4 for these studies. Fig. 7 A shows that one antibody, 8C11, bound to chimeric cadherins N/E-myc, N/E5a-myc, N/E5-myc, and N/E4-myc, but not to N/E3-myc or N/E2-myc. The control anti-myc antibody recognized each chimeric cadherin. When 8C11 was added to BT20N cells in a motility assay, it inhibited motility in a dose-dependent manner, indicating that this antibody did bind near the domain of N-cadherin that was responsible for altering the behavior of these cells (Fig. 7 B). We used the antibody at a dilution of 1:10 to repeat the experiment and to determine if it had any effect on N-cadherin–negative cells. For this experiment, we used smaller filters and counted the number of cells traversing the entire filter. The 8C11 antibody had minimal effect on the motility of N-cadherin–negative cells (Fig. 7 C). In addition, an irrelevant ascites (4A2), used at a dilution of 1:10, had minimal effect on the motility of BT20N or on the motility of untransfected BT20 cells (Fig. 7 C). However, the mAb 8C11 significantly decreased cell motility in the N-cadherin–expressing BT20N cells. Importantly, even at a 1:10 dilution in the mAb 8C11 did not inhibit cell aggregation in N-cadherin–expressing cells (data not shown). In an initial experiment, the 8C11 antibody did not produce a significant change in morphology when applied to N-cadherin–expressing SCC1 cells (data not shown). These results are unexpected and are being further investigated in our laboratory.

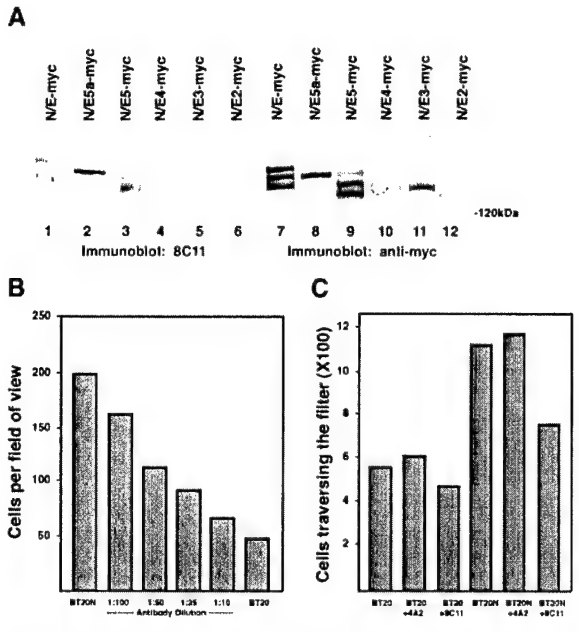

Figure 7. Anti–N-cadherin mAb 8C11 inhibits motility. (A) Extracts of A431D cells transfected with N/E-myc-cadherin (lanes 1 and 7), N/E5a-myc-cadherin (lanes 2 and 8), N/E5-myc-cadherin (lanes 3 and 9), N/E4-myc-cadherin (lanes 4 and 10), N/E3-myc-cadherin (lanes 5 and 11), or N/E2-myc-cadherin (lanes 6 and 12) were resolved by SDS-PAGE and immunoblotted with mAb 8C11 (lanes 1–6) or anti-myc (lanes 7–12). Note, in some cases, we observed various processing variants when transfected cadherins were overexpressed in cells. (B) BT20 cells, which were transfected with N-cadherin (BT20N), were plated on membranes for motility assays in the presence of no antibody or 8C11 ascites at a dilution of 1:10–1:100. Untransfected BT20 cells in the absence of antibody were included as a control. After 24 h, the number of cells traversing the membrane was determined by averaging 10 random fields at 100× magnification. Data are expressed as the number of cells/field. (C) Untransfected BT20 cells or BT20 cells transfected with N-cadherin (BT20N) were plated on membranes for motility assays in the presence of no antibody, irrelevant ascites 4A2 at a dilution of 1:10 or 8C11 ascites at a dilution of 1:10. After 24 h, the number of cells traversing the membrane was determined by counting the entire membrane. Data are expressed as the number of cells traversing the filter.

Discussion

We and others have shown that N-cadherin influences the morphology and behavior of epithelial cells (Islam et al., 1996; Hazan et al., 1997, 2000; Li et al., 1998). These studies implicate N-cadherin in an epithelial to mesenchymal transition in some cells, but not in others. In squamous epithelial cells, expression of N-cadherin results in downregulation of E-cadherin, which is most likely responsible for the change in cellular morphology. In other cells, such as breast cancer cells, expression of N-cadherin does not alter cell morphology, but does alter cellular behavior by inducing a motile phenotype. In breast cancer cells, expression of E-cadherin remains approximately the same when the cells are forced to express N-cadherin. This suggests that even in cells that express abundant E-cadherin, N-cadherin influences cell behavior. N-cadherin is often expressed by motile cells, such as fibroblasts, and a switch from E-cadherin expression to N-cadherin expression occurs when some cells become motile and/or invasive during normal developmental processes (Edelman et al., 1983; Hatta and Takeichi, 1986; Zhou et al., 1997; Huttenlocher et al., 1998). Thus, it is not unexpected that expression of N-cadherin by tumor cells alters cellular morphology and/or behavior.

The extracellular domain of a cadherin promotes cell–cell adhesion, whereas the cytoplasmic domain serves to link the cadherin to the cytoskeleton via interactions with catenins. These cytosolic interactions are critical to the adhesive function of the cadherin. Linkage to the cytoskeleton is necessary to promote strong cell–cell adhesion and to allow organization of the junction itself. In addition, the catenins have been implicated in signaling events that are thought to regulate the strength of the adhesive activity of the cadherin (for review see Gumbiner, 2000). This led us to propose that the cytoplasmic domain of N-cadherin was responsible for increasing the motility of epithelial cells. When we prepared two chimeric cadherins, one comprised of the extracellular domain of N-cadherin linked to the cytoplasmic domain of E-cadherin (N/E-cadherin) and the other comprised of the extracellular domain of E-cadherin linked to the cytoplasmic domain of N-cadherin (E/N-cadherin), we were surprised to find that it was the extracellular domain of N-cadherin that promoted cell motility. The extracellular domain of cadherins is comprised of five repeat regions with EC1 being the most NH_2-terminal. Most of the known activities of cadherins have been mapped to EC1. The best understood examples are those where cadherin molecules interact with other cadherin molecules. Structure determinations (Shapiro et al.,1995; Nagar et al., 1996; Tamura et al., 1998; Pertz et al., 1999) and biochemical characterization (Nose et al., 1990; Ozawa et al., 1990; Ozawa and Kemler, 1990; Koch et al., 1997; Shan et al., 2000) have demonstrated that EC1 is the site of the adhesion interface. Data from several laboratories have suggested that cadherins are displayed on the surface of cells as dimers (Shapiro et al., 1995; Brieher et al., 1996; Chitaev and Troyanovsky, 1998; Takeda et al., 1999; Shan et al., 2000). Although several differing pictures exist as to how these cis (also called lateral) dimers form and are maintained, the data point to EC1 and EC2 of the cadherins as playing major roles.

In some instances, it has been shown that cadherins can promote cell–cell adhesion via heterophilic interactions, for example N-cadherin can bind to R-cadherin (Inuzuka et al., 1991), B-cadherin can bind to L-CAM (Murphy-Erdosh et al., 1995), and cadherin-6B can bind to cadherin-7 (Nakagawa and Takeichi, 1995). Recently, Shimoyama et al. (2000) examined eight different type II cadherins and frequently observed interactions between L cells transfected with different cadherins. Another recent study showed that, in L cells expressing both N- and R-cadherins, the two cadherins formed cis heterodimers that functioned in cell adhesion (Shan et al., 2000). In this latter case, it was the NH_2 terminus of the cadherins that played a role in the formation of the cis heterodimers. It will be interesting to determine if other pairs of cadherins shown to mediate heterophilic cell–cell adhesion are able to form cis

heterodimers and what parts of the cadherins are involved. Here, we have shown that the ability of N-cadherin to promote cell motility resides in EC-4. Thus, this activity is distinct from the adhesive function of the cadherin.

In addition to the interaction of cadherins with themselves, various other interacting proteins have been described. The bacterium *Listeria monocytogenes* has been shown to use E-cadherin as a receptor. InlA, a surface protein on the bacterium, binds to E-cadherin. Lecuit et al. (1999) showed that changing a single amino acid in EC1 of E-cadherin (proline-16 of EC1) eliminated the binding of InlA and dramatically compromised internalization of *Listeria* by cells. In addition to being a target for *Listeria*, E-cadherin is the only cadherin that is known to be an integrin ligand. Integrin $\alpha_E\beta_7$ binds EC1 of E-cadherin, and glutamate-31 of EC1 plays a critical role in the interaction (Karecla et al., 1996). Since EC1 of cadherins has been shown to play a major role in their biological activities, all of the chimeras used here retained the intact EC1 of N-cadherin.

Although most activities have been mapped to the NH_2-terminal domains, there are several reports suggesting roles for EC3, EC4, and EC5 in cadherin adhesion. Zhong et al. (1999) have characterized a mAb (AA5) recognizing EC5 of C-cadherin that activates adhesion, perhaps by changing the cadherin's organization or altering its interaction with other cellular factors. Sivasankar et al. (1999) have studied the biophysical characteristics of adhesion mediated by layers of oriented recombinant C-cadherin ectodomains. They concluded that complete interdigitation of antiparallel ectodomains (i.e., where EC1 of one molecule interacted with EC5 of the antiparallel partner, EC2 interacted with EC4 of the partner, etc.) gave the strongest interactions. Their data also suggested that ratcheting the molecules one EC domain further apart (such that EC1 interacted with EC4 of its antiparallel partner, etc.) also resulted in an adhesive interaction. In addition, Troyanovsky et al. (1999) have reported that EC3 and EC4 of E-cadherin can mediate cis dimerization under some conditions.

A series of papers from Lilien's laboratory (for review see Lilien et al., 1999) have suggested that in neural retina cells, the ectodomain of N-cadherin is stably associated with and is a substrate for the cell surface enzyme *N*-acetylgalactosaminyphosphotransferase. The interaction of neurocan, a chondroitin sulfate proteoglycan, with *N*-acetylgalactosaminyphosphotransferase results in inhibition of N-cadherin–mediated cell adhesion. However, the site(s) on N-cadherin where this interaction takes place is unknown.

Investigators have suggested that N-cadherin can interact with and activate fibroblast growth factor receptors (FGFR) in neurons (Doherty and Walsh, 1996) and ovarian surface epithelial cells (Peluso, 2000). In the ovarian surface epithelial cell system, it has been reported that N-cadherin and FGFR coimmunoprecipitate. To date, this interaction has not been substantiated by other labs. Our laboratory recently showed that N-cadherin–mediated cell motility of breast cancer cells can be decreased by an inhibitor of the FGF-mediated signal transduction pathway, which has been characterized by the Walsh and Doherty labs (Nieman et al., 1999a). In addition, Hazan et al. (2000) showed that FGF caused a dramatic increase in motility in N-cadherin–expressing cells. The FGFRs contain an HAV sequence (Byers et al., 1992) that has been proposed to interact with EC4 of N-cadherin. It is interesting to note that the 69–amino acid segment of N-cadherin we have identified here includes the sequences proposed by Doherty and Walsh to interact with the FGFRs. The structure of a portion of FGFR1 bound to FGF2 has been determined (Plotnikov et al., 1999). The histidine and valine side chains of the HAV sequence in FGFR1 were involved in intradomain contacts and, thus, appear to be unavailable for interacting with partner molecules. Thus, the precise role the FGFR plays in N-cadherin–dependent cell motility is still unknown and it is not clear at this time whether N-cadherin and the FGFR directly interact with one another.

Many studies have shown that N-cadherin promotes cell motility that is dependent on the adhesive function of N-cadherin. The best studied example is that of N-cadherin–dependent neurite extension. In vitro experiments have demonstrated that N-cadherin promotes neurite outgrowth as a purified protein or when it is expressed by transfected cells. Importantly, antibodies that block the adhesive function of N-cadherin block this outgrowth, and it has been suggested that N-cadherin may guide axonal outgrowth in vivo (for review see Grunwald, 1996). In addition, Hazan et al. (1997) suggested that N-cadherin–mediated motility of tumor cells might be due to the interactions of N-cadherin–expressing epithelial cells with N-cadherin–expressing stromal cells. In contrast, the studies presented here, using the 8C11 mAb, provide evidence that N-cadherin may influence the motility of epithelial cells in a manner that is independent of cell–cell adhesion.

Since the 69–amino acid portion of N-cadherin can influence epithelial cell morphology and motility, we compared this portion of human N-cadherin to other cadherins. In this region, mouse and rat N-cadherin are identical to human N-cadherin, whereas 78% of the amino acids in human R-cadherin are identical. The corresponding region of human E-cadherin contains 70 amino acids and is 54% identical to N-cadherin. To further investigate the role this portion of N-cadherin plays in cell motility, we produced a mAb that binds near EC-4 of N-cadherin. When applied to cells in a motility assay, this antibody inhibited cell motility in N-cadherin–expressing cells, but not in N-cadherin–negative cells. In addition, this antibody inhibited motility without inhibiting cell–cell aggregation, providing further evidence that adhesion and motility are two separate properties of the extracellular domain of N-cadherin. It is important to remember that all the chimeras used here were full-length cadherins. Studies are in progress to determine if truncated cadherins can influence cell motility.

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Cadherin Junctions in Mammary Tumors

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Cadherins are the transmembrane component of adherens junctions found between interacting cells in tissues. The cadherins bind cells to one another in a specific manner and link to the actin cytoskeleton through intracellular catenins. In addition to promoting strong cell-cell adhesion, cadherins appear to initiate and modify intracellular signaling pathways. The loss of E-cadherin function in epithelial cells is thought to be an important step in tumorigenesis. Moreover, anomalous expression of inappropriate cadherins in epithelial cells alters their behavior and may contribute to the tumorigenic phenotype. For breast cancer the decreased expression of E-cadherin alone may have limited value as a prognostic indicator; however, examining the repertoire of cadherins and catenins expressed by tumors may provide useful prognostic information.

KEY WORDS: Cadherin; cancer; motility; invasion.

OVERVIEW OF CADHERINS AND CATENINS

Cadherins

The cadherins are members of a large family of transmembrane glycoproteins that mediate calcium-dependent homotypic cell-cell adhesion. They play important roles in embryonic development and in the maintenance of normal tissue architecture. The so-called classical cadherins, including E-, P-, and N-cadherin, are the focus of this review. They are the transmembrane component of the adherens junction and are composed of three segments: (1) an extracellular domain responsible for homotypic cadherin-cadherin interaction, (2) a single pass transmembrane domain, and (3) a highly conserved cytoplasmic domain that is linked to actin filaments and thus serves to connect the cell surface to the cytoskeleton (Fig. 1). The ability of cadherins to simultaneously self-associate and to interact with the actin cytoskeleton achieves both the cellular recognition required for cell sorting during embryogenesis and the strong cell-to-cell adhesion needed to form and maintain tissues. In addition, mounting evidence suggests that cadherin adhesion plays a signaling role during cell growth and differentiation. Because of the importance of cadherins to cell recognition, adhesion, and signaling, disruption of cadherin function has significant implications for the development and behavior of tumors.

The extracellular cadherin domain is directly responsible for the protein-protein interactions that mediate cell-cell adhesion. The earliest evidence supporting this idea came from studies demonstrating that antibodies produced against the extracellular domain of cadherins inhibit cell-cell adhesion. The extracellular domain can be divided into five "extracellular cadherin structural domains" (EC), each of which consists of approximately 110 amino acids and contains the conserved motifs LDRE, DXNDN and DXD. EC-1 is the most N-terminal domain and is responsible for adhesive activity. The binding sites for most monoclonal antibodies that inhibit the adhesive function of cadherins have been mapped to EC-1, which also contains the HAV tripeptide implicated in adhesion. Synthetic peptides containing an

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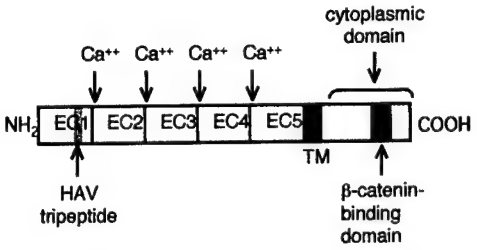

Fig. 1. Structure of classical cadherins. The cadherin is composed of an extracellular domain (EC), a single pass transmembrane domain (TM) and a cytoplasmic domain. The extracellular domain contains five EC repeats, each bridged by Ca^{++} ions. EC1 contains the HAV tripeptide and is responsible for cell-cell adhesion. The cytoplasmic domain contains the β-catenin-binding site that serves to link the cadherin to the actin cytoskeleton.

HAV sequence, like antibodies to EC-1, can inhibit cadherin-mediated adhesion. Structural studies have shown that the HAV tripeptide and surrounding amino acids mediate cadherin self-association through interaction with a separate set of amino acids within EC-1 of the interacting cadherin on the adjacent cell. Mutations in the N-terminus of cadherins, or deletion of EC-1, result in molecules that fail to promote cell adhesion (reviewed in 1–5).

Cells expressing different members of the cadherin family segregate from one another when mixed together *in vitro*. It has been proposed that this preferential binding of cadherins plays an important role in the sorting activities of embryonic cells. Interestingly, the binding specificity of cadherin molecules also maps to EC-1. When the amino-terminal regions of E-cadherin were replaced with those of P- or N-cadherin, the chimeric molecules displayed P- or N-cadherin specificity, respectively. Thus, EC-1 of the classical cadherins is responsible not only for the adhesive activity but also for cadherin specificity, i.e., the homotypic interaction of cadherins such as E-, P-, and N- cadherin. As a result of this property, altered expression of cadherins by tumor cells may induce new or inappropriate cell-cell adhesion and subsequently altered or abnormal intracellular signaling (6; reviewed in 1, 3–5).

A number of studies suggest that cadherins function in activities other than cell-cell recognition and adhesion. In fact, there is growing evidence that multiple intracellular signaling events are initiated or modulated by cadherin adhesion (4). For example, some years ago Walsh and Doherty showed that N-cadherin promotes neurite outgrowth in cultured neurons. They proposed that this cell response resulted from an interaction between N-cadherin and the fibroblast growth factor receptor (FGFR), resulting in FGFR signal transduction in the absence of ligand (reviewed in 7). More recently, two laboratories, including our own, have presented data implicating a role for N-cadherin in altering the morphology and migration of tumor cells, including human mammary carcinoma cells (8, 9).

Catenins

Cadherins are not bound directly to the actin cytoskeleton but rather are connected to it indirectly via a group of proteins collectively known as the catenins (Fig. 2). The catenins were first identified as proteins that co-immunoprecipitated with cadherins, and were named α-catenin, β-catenin and γ-catenin according to their mobility on SDS-PAGE. Alpha-catenin is a 102 kDa protein that associates with the cadherin indirectly through its interaction with β-catenin or γ-catenin. Beta-catenin is a 95 kDa protein that shares about 65 percent identity with γ-catenin, an 82 kDa protein also named plakoglobin. Beta-catenin and plakoglobin associate directly with the cadherin in a mutually exclusive way and can substitute for one another in the cadherin-catenin complex. Thus, the cell-cell adherens junction is a plasma membrane structure composed of transmembrane cadherins associated directly with either β-catenin or plakoglobin, which associates with α-catenin. In turn, α-catenin mediates the interaction between the cadherin-catenin complex and the actin cytoskeleton. Additional proteins that interact with catenins to provide a link to the actin cytoskeleton include ZO1, α-actinin and vinculin (10, 11; reviewed in 3, 4).

Catenins act to regulate the adhesive function of cadherins in addition to the structural role they play in the adherens junction. For example, phosphorylation of β-catenin in Src-transformed cells appears to contribute to the non-adhesive phenotype of these cells (Reviewed in 12). In addition, β-catenin (perhaps also γ-catenin) serves as an important signaling molecule, playing a critical role in both tissue patterning during development and maintaining the normal cellular phenotype (13–15). The signaling functions of β- catenin result from interactions with transcription factors of the LEF/TCF family and with receptor tyrosine kinases (reviewed in 16).

An additional catenin, $p120^{ctn}$, is emerging as a factor important for regulating cadherin adhesive activity. Originally identified as a Src substrate, $p120^{ctn}$ has subsequently been shown to interact directly with

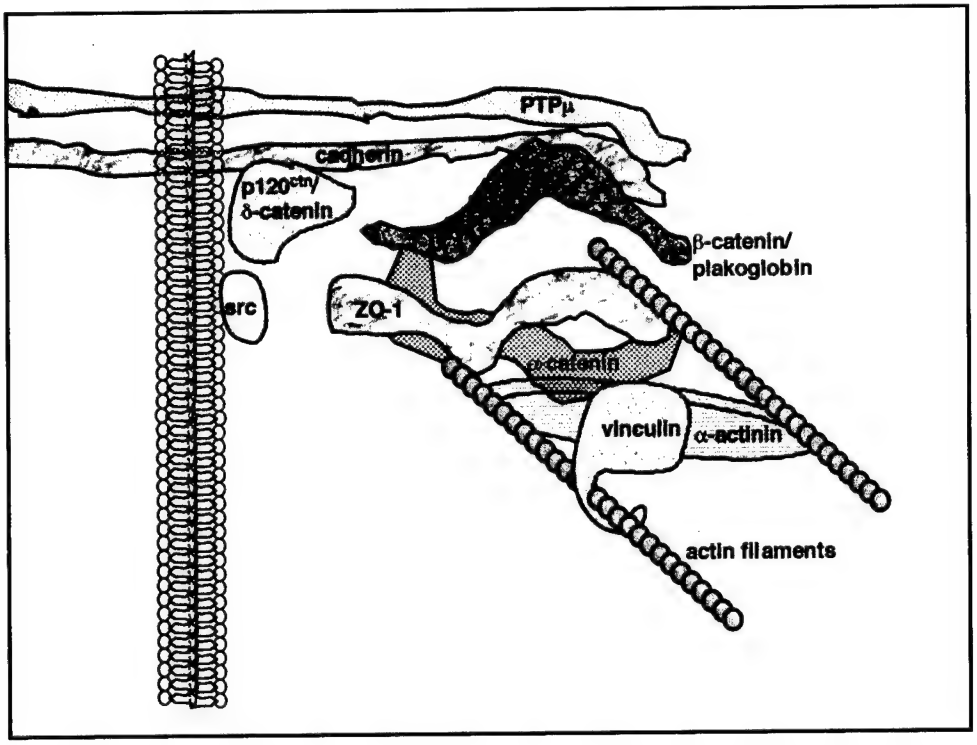

Fig. 2. Linkage of a cadherin to the actin cytoskeleton. The cadherin cytoplasmic domain binds to β-catenin or plakoglobin, which in turn binds to α-catenin to link the cadherin to the cytoskeleton. Other molecules involved include ZO1, vinculin and α-actinin. $P120^{ctn}$ binds to the juxtamembrane domain of the cadherin cytoplasmic domain, and protein tyrosine phosphatase μ (PTPμ) binds to the extreme C-terminus of the cadherin. These two proteins may modulate adhesive activity of the adherens junction. In addition, Src is situated at the membrane where it also may contribute to the regulation of cadherin function.

the cytoplasmic domain of cadherins (Fig. 2). $P120^{ctn}$ binds to the juxtamembrane region and has been implicated in cadherin clustering and cell motility. It has been proposed that $p120^{ctn}$ influences the strength of cadherin-mediated cell-cell adhesion positively or negatively, depending on the cell type and $p120^{ctn}$ phosphorylation state (reviewed in 12). $P120^{ctn}$ may modulate cell adhesion by influencing the organization of the actin cytoskeleton or by influencing the activity of RhoA, a small GTPase involved in actin cytoskeletal dynamics (17–21). Phosphatases such as PTPμ bind to the extreme C-terminus of cadherins and also may play a role in regulating their function (reviewed in 22). Together, the catenins that associate with the cytoplasmic domain of cadherins can influence the adhesive function of the cadherin in a dynamic manner. This flexibility in the strength of adhesion is important to embryogenesis and tissue repair, but also may contribute to tumor formation and aggression.

ROLE OF CADHERINS IN TUMORIGENESIS

Involvement in Tumorigenesis and Invasion

The vast majority of studies implicating cadherins in tumorigenesis and invasion have focused on E-cadherin, because E-cadherin is the major cadherin involved in epithelial cell-cell adhesion, and the majority of human cancers originate from epithelial cells. E-cadherin is the transmembrane component of the adherens junction in most epithelial cells. The adherens junction functions to maintain the normal phenotype of these cells and is responsible for the strong cell-cell adhesion that promotes epithelial polarity and prevents epithelial cells from migrating away from their appropriate location. A role for E-cadherin in limiting invasion of tumor cells and acting as a suppressor of invasion has been accepted readily. Early studies showed that inhibiting E-cadherin activity with function-perturbing antibodies, the

morphology of normal kidney epithelial cells and conferred upon them the ability to invade both collagen gels and embryonic chicken heart tissue. In addition, invasive carcinoma cells have been converted to a noninvasive phenotype by transfecting the cells *in vitro* with a cDNA encoding E-cadherin (reviewed in 23–25). Moreover, an important *in vivo* study showed that downregulation of E-cadherin activity using a dominant-negative form of E-cadherin in a mouse model system resulted in transition of a well-differentiated pancreatic β-cell adenoma to an invasive carcinoma (26).

Downregulation of E-cadherin function can occur via multiple mechanisms, including gene mutations. A number of studies have reported a correlation between mutations in the gene encoding human E-cadherin (CDH1) and the presence of invasive carcinoma of bladder, breast, endometrium, liver, lung, ovary, prostate, and thyroid (reviewed in 27). Mutations include missense mutations, splice site mutations and truncation mutations. Frequently these mutations occur in combination with loss of heterozygosity of the wild-type allele. In addition to its role as an invasion suppressor, E-cadherin appears to function as a true tumor suppressor gene. Individuals with germ line mutations in E-cadherin are susceptible to sporadic diffuse gastric cancers and sporadic breast cancer (reviewed in 27). Although E-cadherin mutations can be detected in some tumors, the majority of cases of malignant carcinomas appear to lack mutations in this gene. However, it is thought that the tumor cells must downregulate the activity of the E-cadherin/catenin complex in order to invade surrounding tissues.

Aside from mutations in the gene, the mechanisms whereby tumor cells decrease the expression of E-cadherin are poorly understood. Expression levels of E-cadherin can be modulated by a number of proteins shown to play a role in tumorigenesis, including PAX2, RB, c-myc, ERBB2, and the LEF1/β-catenin transcription factor complex. In addition, hypermethylation of CpG islands in the E-cadherin promoter has been demonstrated in certain human carcinomas (reviewed in 23). Thus, it is likely that downregulation of E-cadherin expression in tumors can be achieved by a variety of different mechanisms.

An alternative mechanism for inactivating the adhesive function of the adherens junction in tumor cells is to disrupt the connection between the cadherin and the cytoskeleton. Decreased cadherin adhesive function can result from mutations in genes encoding the catenins. For example, mutations in β-catenin that disrupt its binding to E-cadherin result in a nonadhesive phenotype. In addition, mutations in the gene that encodes α-catenin effectively inactivate E-cadherin function by not allowing the cadherin complex to associate with the cytoskeleton. Moreover, cells deficient in catenins display normal adhesiveness when transfected with cDNAs encoding functional catenins. Analysis of human tumor sections has demonstrated reduced expression of catenins in some tumors. For example, reduced expression of α-catenin has been associated with gastric cancer, colon cancer and prostate cancer, whereas reduced expression of β-catenin has been shown in esophageal, gastric, and colon tumors. In addition, both α- and β-catenin have been shown to be important prognostic factors in some cancers (reviewed in 25, 28).

The function of the cadherin/catenin complex may be altered during tumorigenesis by phosphorylation. For example, it has been recognized for some time that Src-transformed epithelial cells display decreased cell-cell adhesion. While the cadherins are poor substrates for tyrosine kinases, β-catenin, plakoglobin and p120^{ctn} are highly phosphorylated on tyrosine in Src-transformed epithelial cells. There is compelling evidence that phosphorylation of catenins plays a role in regulating the adhesiveness of the cadherin complex, although the mechanism of regulation remains controversial (reviewed in 12). In addition to its role in the adherens junction, β-catenin is a component of the wnt signal transduction pathway shown to play a role in the development of a number of different tumor types, in particular tumors of the colon. The wnt pathway is highly regulated and involves multiple proteins, including GSK3-β, axin, conductin, TCF and APC. Mutations in β-catenin or other components of the pathway that result in accumulation of cytosolic β-catenin lead to increased interaction of β-catenin with the transcription factor TCF, which enhances transcription of β-catenin-regulated genes and tumorigenesis (reviewed in 16).

Finally, recent studies from our laboratory and other laboratories have shown that expression of an inappropriate cadherin in epithelial cells is yet another way that tumor cells can alter their adhesive function (8, 9, 29). In some cases, this may be due to downregulation of E-cadherin upon expression of the inappropriate cadherin (30, 31). In other cases, mesenchymal cadherins can have a direct and dominant influence on the phenotype of epithelial cells, despite their continued expression of E-cadherin (8, 9, 31). In short, regardless of the mechanism, disrupting the function of the E-cadherin/catenin complex

in epithelial cells favors the formation of invasive tumorigenic cells.

Cadherins as Tumor Markers

Cadherins have proven to be useful tumor markers both for identifying the cellular origin of a tumor and for denoting changes in the normal cell phenotype. The cadherin family includes a number of distinct members that are differentially expressed throughout embryonic development. During morphogenesis, the expression of distinct members of the cadherin family determines coalescence of cells into specialized tissues (reviewed in 1). For example, E-cadherin is expressed primarily by epithelial cells; N-cadherin by nerve cells, developing skeletal muscle, myocardial cells, and pleural mesothelial cells; M-cadherin primarily by skeletal muscle; P-cadherin by the basal layer of skin, myoepithelial cells of the mammary gland, and basal cells of the prostate; R-cadherin by retinal cells; and OB-cadherin (or cadherin-11) by osteoblasts and fibroblasts. In addition, the cadherin family includes a large number of minor members and many, if not most, cells express more than one cadherin (reviewed in 5). Thus, the repertoire of cadherins expressed by a cell may serve to identify its tissue of origin, and cadherin antibodies can be used to distinguish tumor types that are difficult to diagnose by classical histological techniques. For example, N-cadherin has been shown to be an effective marker to distinguish malignant mesotheliomas from peripheral lung adenocarcinomas (32).

Since E-cadherin is the major cadherin expressed by epithelial cells and it is accepted to be both an invasion and tumor suppressor, the loss of E-cadherin can be useful as a prognostic indicator for some tumors. Reduction of the expression of E-cadherin has been associated with lack of cohesiveness, higher malignant potential and invasiveness in epithelial neoplasms of the colon, ovary, stomach, pancreas, lung, breast, head and neck and other sites (32). Excellent antibodies are available commercially that recognize cadherins and catenins in paraffin sections, making analysis of these proteins possible using established immunohistochemical techniques (see Table I).

Recent studies from several laboratories, including our own, suggest that cadherins not normally expressed by epithelial cells may be inappropriately expressed by some epithelial tumors. This aberrant expression can significantly alter the behavior of the epithelial cells (8, 9, 29, 30, 33). For example, when epithelial cells express N-cadherin or cadherin-11, the cells display increased cell motility and invasion.

Table I. Antibodies for Immunohistochemical Localization of Cadherins and Catenins in Formalin-Fixed, Paraffin-Embedded Breast Tissue

Cadherin	Location in normal nonlactating tissue	Location in tumors	Antibody*/vendor
E-Cadherin	Plasma membrane of luminal epithelial cells	Present, reduced, or missing; membrane and/or cytoplasmic	HECD-1/Zymed
P-Cadherin	Plasma membrane of myoepithelial cells	Absent of present; membrane and/or cytoplasmic	Clome 56/BD Transduction Labs
N-Cadherin	Not detected but likely expressed by mesenchymal cells	Absent or present; membrane and/or cytoplasmic; may be focal	3B9/Zymed
β-Catenin	Plasma membrane of epithelial and myoepithelial cells	Generally present but may be missing	CAT-5H10/Zymed Clone 14/BD Transduction Labs
α-Catenin	Plasma membrane of epithelial and myoepithelial cells	Missing in some cancers	αCAT-7A4/Zymed C2081/Sigma
Palkoglobin	Plasma membrane of epithelial cells	Missing in some cancers	4F11/Zymed
p120^{ctn}	Plasma membrane and cytoplasm	Missing in some tumors	15D2/Zymed

*Only highly specific antibodies suitable for staining of formalin-fixed, paraffin-embedded tissues are listed. Heat-induced antigen retrieval is required prior to antibody staining.

Thus, it is important to know the cadherin profile of both normal and tumor tissue, as the presence of E-cadherin expression does not always indicate a nonaggressive phenotype. For example, ovarian carcinomas arise from the surface epithelium which normally expresses N-cadherin. During malignant progression these cells switch from N-cadherin to E-cadherin expression (34). Hence, E-cadherin may serve as a marker for ovarian cancer. As additional cadherins are identified as being inappropriately expressed by tumors, these cadherins, in addition to the loss of E-cadherin, may become useful for predicting the malignant behavior of a tumor.

Cadherin Fragments in Body Fluids

Cadherins are transmembrane proteins, with the majority of the protein extending externally from the cell surface. One mechanism for inactivating cadherins, and apparently a normal part of their turnover in cells, is cleavage of the extracellular domain, with soluble cadherin fragment being released into the extracellular space. Soluble forms of E- and P-cadherin have been detected in serum (35, 36). Increased proteolytic activity in tumors could result in increased release of the cadherin extracellular domain, leading to increased levels of circulating cadherin. Thus, it has been suggested that detection of cadherin fragments in serum may be a useful, noninvasive tool for diagnosis. A soluble fragment of E-cadherin was shown to be significantly elevated in patients with metastatic melanoma but was also elevated in patients with nonneoplastic skin disorders (37). In addition, serum levels of E-cadherin were shown by some studies to be increased in patients with gastric cancer and colorectal cancer, but other studies contradict these results. We analyzed patients undergoing breast surgery for their levels of both E- and P-cadherin, but could detect no differences between patients with or without a malignancy. It is likely that unless the tumor burden is large an elevation in serum levels of cadherin will not be detected (36).

CADHERINS AND BREAST CANCER

E-Cadherin and Catenins

In normal breast tissue E-cadherin is expressed by the luminal epithelial cells, and is found concentrated at cell-cell borders. As is the case of other carcinomas, breast cancer cells sometimes exhibit reduced or missing E-cadherin expression, or function. A number of cell lines isolated from human breast cancers were found to be negative for E-cadherin (8, 38). The loss of E-cadherin expression by breast cancer cells appears to involve multiple mechanisms, including complete or partial gene deletion, promoter inactivation by methylation, and chromatin rearrangement (38, 39). Forced expression of E-cadherin in E-cadherin-negative breast cancer cells suppresses their metastasis in a mouse model system (40, 41), suggesting that E-cadherin may serve as an invasion suppressor in breast cancer. For breast cancer cells that continue to express E-cadherin, *in vitro* studies have shown that the E-cadherin level or activity can be modulated positively by exogenous agents such as tamoxifen (42), adriamycin (43), and insulin-like growth factor I (44), or negatively by agents such as alcohol (45). Thus, *in vitro* studies suggest that the activity of E-cadherin may be modulated dynamically in breast tumors.

A large number of studies have employed immunohistochemical methods to evaluate E-cadherin expression in tumors obtained from breast cancer patients. In general these studies document a loss of E-cadherin in the majority of lobular carcinomas, which represent a minority of breast cancers (46–50). E-cadherin loss in lobular carcinomas results from loss of heterozygosity of the E-cadherin gene (CDH1) together with mutations, including frameshift, nonsense, or splice mutations, scattered over the entire coding region (27, 50). It has been suggested that loss of E-cadherin is an early event in lobular breast cancer, occurring even in lobular carcinoma *in situ* (51). In fact, lobular breast carcinomas have been associated with germ line mutations in E-cadherin (52).

In contrast to lobular carcinomas, most ductal carcinomas retain E-cadherin expression, although the level may vary (46–49, 53, 54). Unfortunately, it is difficult to directly compare the many studies reported in the literature because of differences in patient populations, tissue preparation, antibodies used, and immunohistochemical methods. However, in general E-cadherin expression is higher in well-differentiated and less-invasive tumors, as opposed to poorly differentiated or invasive tumors (55, 56). While E-cadherin expression has been correlated with a high degree of tumor differentiation, it has been found to be independent of lymph node status and tumor size (57).

E-cadherin expression also has been evaluated with respect to breast cancer metastasis. One study showed that axillary lymph node metastases were

completely missing only in breast cancer patients with preserved E-cadherin expression in the primary tumor (58). Another study showed a correlation between reduced plasma membrane E-cadherin staining (or presence of cytoplasmic staining) in the primary tumor and lymph node metastasis (59). These studies support an anti-invasion role for E-cadherin in human breast cancer. However, analysis of E-cadherin in metastatic tissue reveals that its expression frequently differs from that of the primary tumor (60). One study showed that the majority of bone micrometastases in breast cancer patients were E-cadherin positive (61).

It is possible that the loss of E-cadherin expression or function promotes tumor cell invasion, whereas its reexpression may promote survival of intravascular cancer cells and tumor cells at metastatic sites. This dynamic modulation of cadherin expression during tumor invasion and metastasis may result from the cells responding to distinctive signals in their environment. Scattering cancer cells invading the relatively solid matrix of the breast tissue must change their characteristics for surviving in the flowing liquid environment of lymph and blood vessels. Supporting this idea, histological sections of breast tumors show scattered extravascular tumor cells. However, in vessels, the cancer cells tend to form tight clusters. Interestingly, the levels of E-cadherin expression actually are increased in intravascular breast cancer cells when compared to extravascular cancer cells (62). Such dynamic changes in the expression of E-cadherin could be induced by unstable methylation of the E-cadherin promoter (63) or could be due to posttranscriptional regulation of the cadherin/catenin/cytoskeleton complex in response to environmental signals.

Most importantly, a number of studies have evaluated the usefulness of E-cadherin expression with respect to survival of breast cancer patients. Several studies indicate that reduced E-cadherin expression is an independent indicator of poor survival, particularly in node-negative patients (49, 64–67). However, other studies have found E-cadherin to be of little use in predicting clinical behavior of breast cancer patients (54, 68, 69). Indeed, one study even noted that strong E-cadherin immunostaining correlated with poor survival (70). In short, there currently are conflicting opinions about the prognostic value of altered immunohistochemical patterns of E-cadherin. Thus, the usefulness of E-cadherin alone as a predictor of clinical behavior for breast cancer patients is still under debate.

Breast cancers with reduced or missing E-cadherin also can exhibit reduced catenins. This is not unexpected since catenins bind to and are stabilized by cadherins. In turn, full functional activity of E-cadherin is dependent on catenin binding. One study indicated that α-catenin was reduced or lost even more frequently than E-cadherin in breast cancers (71). The authors suggested that α-catenin may serve as a marker for disturbances in the adhesive function of E-cadherin. Another study showed that reduced expression of E-cadherin, β-catenin, α-catenin or plakoglobin alone did not correlate highly with metastasis; however, when all four proteins were analyzed as one group, reduction of at least one of these markers did correlate significantly with metastasis in human breast carcinomas (72). While the literature appears to agree about disturbances in immunohistochemical patterns for E-cadherin and catenins in mammary tumors, there is, to date, no consensus about the prognostic value of these changes for breast cancer.

P-Cadherin

In the normal nonlactating breast P-cadherin is expressed by myoepithelial cells underlying the luminal epithelium and by caps cells, which are considered to be a stem cell population in the breast (73). However, P-cadherin was detected in about 30% of mammary carcinoma cell lines (8), suggesting that this cadherin can be expressed by breast epithelial cells. Several studies have investigated the expression of P-cadherin in tumors of the breast. In an early study, P-cadherin was not detected in patients with ductal carcinoma (48). In contrast, a later study found P-cadherin expression in some cases of infiltrating ductal carcinoma, where it was associated with reduced E-cadherin and advanced histologic grade (74). Recently, we showed that approximately half of ductal carcinomas express P-cadherin, whereas it was not detected in lobular carcinomas. Most importantly, our study showed that P-cadherin expression is associated significantly with poor survival and constitutes an independent prognostic predictor of survival for breast cancer patients. In fact, P-cadherin expression was a better indicator of clinical outcome than alterations in the expression of E-cadherin or catenins. In a study designed to determine if the level of soluble P-cadherin in serum might be elevated for patients with P-cadherin-positive tumors and therefore serve as a useful clinical marker, we found no correlation between soluble P-cadherin and breast cancer (36).

H-Cadherin

H-Cadherin differs from E-, P-, and N-cadherin in that it lacks an intracellular domain. However, H-cadherin does have adhesive properties. The H-cadherin gene has been localized to chromosome 16q24, a region that exhibits loss of heterozygosity in a number of sporadic breast cancer (75). In addition, its expression was reported to be reduced in human breast carcinoma cell lines and breast cancer tissue (76). Transfection of H-cadherin into a breast cancer cell line was shown to prevent invasiveness and tumor growth in nude mice (77). Whether or not H-cadherin plays a significant role in human breast cancer remains to be seen.

N-Cadherin and Cadherin-11

Although a number of studies have indicated that loss of E-cadherin function in breast epithelial cells results in an invasive phenotype, important exceptions have been seen. Sommers *et al.* (78) showed that transfection of E-cadherin into two invasive breast cancer cell lines did not decrease their invasive capacity, in contrast to studies by other laboratories. We later showed that these two cell lines express N-cadherin and subsequently demonstrated that a number of invasive breast cancer cell lines express N-cadherin (8). In addition, we showed that transfection of N-cadherin into E-cadherin positive breast cancer cells altered their characteristics, converting them from noninvasive, nonmotile cells to invasive motile cells even though they continued to express high levels of E-cadherin. Hazan *et al.* (9) extended our studies to demonstrate that human breast cancer cells transfected with N-cadherin are metastatic in nude mice. Studies from the Byers laboratory have shown that human breast cancer cells expressing cadherin-11 also are invasive, suggesting that cadherin-11 may act in a manner similar to N-cadherin to promote cell motility in breast epithelial cells (29). Two different factors have been proposed to contribute to this alteration in behavior. First, a change in cadherin expression from E-cadherin to a cadherin expressed by stromal cells may increase the ability of the tumor cell to break away from the normal surrounding epithelial cells and interact with the stromal cells. Second, expression of an inappropriate cadherin may initiate inappropriate signal transduction pathways that result in increased cell motility. It is possible that both of these ideas are correct and moreover, that future studies will identify additional inappropriately expressed cadherins that influence the behavior of human breast epithelial cells.

PERSPECTIVES AND FUTURE RESEARCH

It is clear from a growing body of literature that cadherin/catenin cell-cell adhesion complexes dramatically effect the behavior of cells. They can initiate intracellular signaling pathways that alter the growth, migration, invasion, and differentiation of cells (4). Cadherin expression or activity can be altered irreversibly by loss of heterozygosity and mutations, and reversibly by growth factors and cytokines. In turn, cadherins can enhance or suppress the activity of growth factors. Thus, the presence or absence of cadherins has a marked effect on cells.

In general the loss of E-cadherin from epithelial cells promotes their dedifferentiation, growth, and invasion. Thus, the loss of E-cadherin in breast cancers, particularly in node negative patients, can signify a poor prognosis. The use of E-cadherin alone as a tumor marker has less value than when it is coupled with other markers (66), including catenins and other cadherins. However, in many cases the presence or absence of E-cadherin seems to have little value for predicting clinical outcome. This conclusion might reflect a number of variables in published studies, including selection of tumors, demographics of the study population, and differences in the E-cadherin antibody and staining technique used. Larger and more carefully controlled studies are needed to definitively determine the usefulness of E-cadherin and catenins as prognostic indicators for breast cancer.

The expression of cadherin family members not normally found in mammary epithelial cells may have more prognostic value. P-cadherin-positive tumors correlate with poor patient survival, and P-cadherin constitutes an independent prognostic predictor for breast cancer (54). N-cadherin-positive mammary epithelial cells have increased migration and invasion *in vitro*, and enhanced metastasis in a mouse model system (8, 9).

An altered repertoire of cadherin family members in breast cancer cells is predicted to change their behavior compared to normal epithelial cells. Inappropriate intracellular signaling can be activated. For example, N-cadherin may initiate intracellular signaling by activating the fibroblast growth factor receptor (8). In addition, cell-cell interactions can be

changed, leading to new intercellular communication and consequently altered intracellular signaling. For example, P-cadherin-positive epithelial cells can form adherens junctions with myoepithelial cells, enhancing gap junction formation and new intercellular communication.

It is not clear what might activate N-cadherin, cadherin-11 and/or P-cadherin expression in a tumor cell whose progenitor does not normally express these cadherins. However, it is likely that changes in the promoter regions of the genes are involved. In addition, growth factors in the tumor environment might stimulate expression of an inappropriate cadherin. Alternatively, changes in DNA methylation or acetylation in the tumor cells might trigger inappropriate cadherin expression.

A comprehensive understanding of the role that cadherins and catenins play in breast cancer progression is important for diagnosing and treating the disease. For example, if inappropriate cadherin expression enhances the aggressiveness of breast cancers, and the mechanism for the inappropriate cadherin expression is deciphered, then it may be possible for inappropriate cadherin expression to serve not only as a tumor marker but also as a target for therapy.

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Abstract from a poster presented at the 89th meeting of the American Association for Cancer Research, 1999.

The E-cadherin/catenin complex is important in maintaining an epithelial phenotype and normal tissue architecture. E-cadherin expression is decreased or absent in invasive epithelial tumors from various tissues. This has lead to the hypothesis that down regulation of E-cadherin allows a cell to disassociate from the surrounding cells and to invade the surrounding tissues. In vitro data has shown that not all E-cadherin negative breast carcinoma cell lines are invasive. In the present study, we examine the expression of N-cadherin in breast carcinoma cell lines. The E-cadherin negative cell lines that have previously been described as invasive (Hs578t, MDA-MB-435, MDA-MB-436 and BT-549) as well as a new invasive cell line (SUM159) expressed N-cadherin. In contrast, E-cadherin negative cell lines which were not invasive (SKBr-3, MDA-MB-453 and SUM1315m02) did not express N-cadherin. The N-cadherin in these breast cell lines is functional in that it forms a complex with a-catenin and b-catenin as determined by coimmunoprecipitation experiments. These observations suggest that the loss of E-cadherin is not sufficient to confer an invasive phenotype in breast carcinoma cell lines and that the expression of a mesenchymal cadherin (N-cadherin) in breast epithelial cells promotes invasiveness.

Abstract from an invited talk at the Biological Structure and Gene Expression Gordon Conference in 1999.

Decreased expression of E-cadherin has been correlated with increased tumor cell invasion. In some systems, inappropriate expression of a non-epithelial cadherin by an epithelial cell has been shown to down-regulate E-cadherin expression and to contribute to a scattered phenotype. In this study we show that N-cadherin correlates with increased cell motility and invasion in human breast cancer cells. The points we make are:

A. that N-cadherin expression correlates both with invasion and motility in breast cancer cells and likely plays a direct role in promoting motility

B. that decreased expression of E-cadherin does not necessarily correlate with invasion in breast cancer cells

C. that forced expression of E-cadherin in invasive, N-cadherin positive cells does not reduce their motility or invasive capacity

D. that forced expression of N-cadherin in non-invasive, E-cadherin-positive cells produces an invasive cell even though these cells continue to express high levels of E-cadherin

E. that increased invasiveness of N-cadherin-expressing breast carcinoma cells is likely not due to interactions with stromal cells.

Abstract from an invited talk at the American Society for Cell Biology Meeting in 1999.

E-cadherin is a transmembrane glycoprotein that mediates calcium-dependent, homotypic cell-cell adhesion and plays a role in maintaining the normal phenotype of human breast epithelial cells. Decreased expression of E-cadherin has been correlated with increased invasiveness of breast cancer. In other systems, inappropriate expression of a non-epithelial cadherin, such as N-cadherin, by an epithelial cell has been shown to down-regulate E-cadherin expression and to increase the invasive potential of the cell. In this study we explored the possibility that expression of non-epithelial cadherins may be correlated with increased invasion in human breast cancer cells. We showed that up-regulation of inappropriate cadherins, rather than down-regulation of E-cadherin, correlates with increased motility and invasion. In most cases breast epithelial cells with reduced E-cadherin expression have turned on the expression of an inappropriate cadherin. However, we found examples of cells with reduced E-cadherin that did not express an inappropriate cadherin. In these cases, the cells were non-motile and non-invasive.

Abstract from an invited talk at the International Bat-Sheva de Rothschild Seminar Weizmann Institute, Rehovot, Israel, 1999.

E-cadherin is a transmembrane glycoprotein that mediates calcium-dependent, homotypic cell-cell adhesion and plays a role in maintaining the normal phenotype of human breast epithelial cells. Decreased expression of E-cadherin has been correlated with increased invasiveness of breast cancer. In other systems, inappropriate expression of a non-epithelial cadherin, such as N-cadherin, by an epithelial cell has been shown to down-regulate E-cadherin expression and to contribute to a scattered phenotype. In this study we explored the possibility that expression of non-epithelial cadherins may be correlated with increased cellular motility and invasion in human breast cancer cells. We present data showing that N-cadherin promotes cell motility and invasion in breast cancer cells; that decreased expression of E-cadherin does not necessarily correlate with invasion in breast cancer cells; that N-cadherin expression correlates both with invasion and motility in breast cancer cells and likely plays a direct role in promoting motility; that forced expression of E-cadherin in invasive, N-cadherin positive cells does not reduce their motility or invasive capacity; and that forced expression of N-cadherin in non-invasive, E-cadherin-positive cells produces an invasive cell even though these cells continue to express high levels of E-cadherin.

Abstract from a poster presented at the Era of Hope DOD Breast Cancer Meeting in 2000.

E-cadherin is a transmembrane glycoprotein that mediates calcium-dependent, homotypic cell-cell adhesion and plays a role in maintaining the normal phenotype of epithelial cells. Decreased expression of E-cadherin has been correlated with increased invasiveness of breast cancer. In other systems, inappropriate expression of a non-epithelial cadherin, such as N-cadherin, by an epithelial cell has been shown to down-regulate E-cadherin expression and to contribute to a scattered phenotype. In this study we explored the possibility that expression of non-epithelial cadherins may be correlated with increased

motility and invasion in breast cancer cells. We show that N-cadherin promotes motility and invasion; that decreased expression of E-cadherin does not necessarily correlate with motility or invasion; that N-cadherin expression correlates both with invasion and motility and likely plays a direct role in promoting motility; that forced expression of E-cadherin in invasive, N-cadherin positive cells does not reduce their motility or invasive capacity; that forced expression of N-cadherin in non-invasive, E-cadherin-positive cells produces an invasive cell even though these cells continue to express high levels of E-cadherin; that N-cadherin-dependent motility may be mediated by fibroblast growth factor receptor signaling; and that cadherin 11 promotes epithelial cell motility in a manner similar to N-cadherin.

Abstract from the Cell Contact and Adhesion Gordon Conference, 2001.
Decreased expression of E-cadherin has been correlated with increased tumor cell invasion. In some systems, inappropriate expression of a non-epithelial cadherin by an epithelial cell has been shown to down-regulate E-cadherin expression and to contribute to a scattered phenotype. In this study we show that N-cadherin correlates with increased cell motility and invasion in human breast cancer cells. The points we make are:

1. that N-cadherin expression correlates both with invasion and motility in breast cancer cells and likely plays a direct role in promoting motility

2. that N-cadherin likely interacts with the FGF receptor

3. that the extracellular domain of N-cadherin is the region that promotes cell motility